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FOREWORD

In explanation of the varied form in which the papers of this Symposium appear in the published proceedings, the following statement is made:

The general title of the Symposium—Perspectives in Development and Growth—was selected to indicate that, whereas in the past the Symposia had in general been organized around a single broad topic, this Symposium was intended to cover several unrelated fields from the point of view of recent developments and promising lines of future advance. On the assumption that more stimulating papers might be obtained if publication were not required, the Executive Committee decided to make the preparation of a manuscript for publication optional with the speakers. By vote of the Society, however, the Secretary was instructed to collect all manuscripts or abstracts of the papers that could be obtained and have them published as the Proceedings of the Symposium.

J. WALTER WILSON
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THE USE OF RADIOACTIVE TRACERS IN BIOLOGY

ROBLEY D. EVANS

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No manuscript was submitted but instead the following bibliography of relevant papers. The two by the speaker, *J. Applied Physics*, Vol. 12, pp. 260-269, and *Electrical Engineering*, Vol. 60, pp. 250-260, contain a general statement of his ideas on the subject.

RANDOM SAMPLE FOR A BIBLIOGRAPHY ON THE USE OF RADIOACTIVE INDICATORS

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AN AUTO-ANTIBODY CONCEPT OF CELL STRUCTURE, GROWTH AND DIFFERENTIATION

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The concept that I wish to present here is that the various macromolecular substances that form the basis of cell structure bear the same sort of relationship to one another as do antigen and antibody. This concept was derived from the results of certain experiments with specific interacting substances of eggs and sperm and supported by various reports in the earlier, as well as the more recent, literature that seemed most readily explicable on such a basis, and by new evidence for the existence within an organism of an agent capable of neutralizing a toxin produced by the same animal. I shall summarize this work briefly and examine some of the implications of the concept for growth and differentiation of cells.

INTERACTING SUBSTANCES OF EGGS AND SPERM

Four specific interacting substances have been obtained from eggs and sperm of marine animals. These comprise: a substance called fertilizin, which F. R. Lillie (41) first adequately described, an antifertilizin derived from sperm (13, 74), an egg-membrane lysin (74), and an antifertilizin from eggs (77). Fertilizin is derived from the gelatinous coat of eggs of the sea-urchin and other animals, and in solution it has the property of causing an agglutination of sperm of the same species. It has been purified and its properties and occurrence in various species of animals have been studied recently by several investigators (6, 19-24, 37, 38, 46-48, 53, 67, 75-80, 85, 86, 91). In the sea-urchin it is found to be a highly acidic protein of low nitrogen content and with some polysaccharide evidently present. It can be converted into a non-agglutinating form and in some species of animals it is found (78) to occur normally in such "univalent" (see 81, 90) form. In one such case (48) it has been possible by special treatment to enable it to agglutinate sperm of the same species. Antifertilizin, which is the substance on the surface of the sperm with which fertilizin reacts, has also been extensively studied and purified (13, 21, 24, 46, 65, 70, 74, 77, 84, 88). It is a protein of relatively

low molecular weight, iso-electric at pH 3. In solution it is capable of agglutinating a suspension of eggs and of forming a precipitation membrane on the gelatinous coat of the egg. Mixtures of solutions of fertilizin and antifertilizin will form a precipitate under the proper conditions. The interaction of fertilizin and antifertilizin exhibits generally a fairly high degree of species specificity. Wide cross-reactivity is sometimes obtained and this may be attributed to distinct heteroagglutinins present in the preparations although it is also possible that the heteroagglutinating action may, in some cases, be a property of the substances themselves (see 82, 87, 89 for fuller discussion of heteroagglutinins). The egg-membrane-lysin was found in sperm of mollusks, but similar agents have also been obtained from sperm of mammals (12, 45, 61, 64) and of amphibians (26) and an analogous substance has been described in sea-urchins (65, 66). It is a protein of greater heat lability than the others. Evidence has been presented (78, 79, 84, 88) that these substances play an important part in the processes of fertilization, facilitating and perhaps being essential for the attachment of sperm to egg, and for the penetration of the latter into the substance of the former. For the present discussion it should be noted that the interaction of these substances is the same as that observed in interactions of antigen with immune antibody. This analogy, which Lillie first made, continues to receive support not only from the general resemblance of the agglutination reactions and lysis in the two cases but in other features such as the specificity of the reactions, the occurrence of zones, and interaction with complement.

It is with the fourth substance, mentioned above, namely the antifertilizin from eggs, that we are particularly concerned in the present account. Lillie (42) had postulated the existence of such a substance, within the egg, that could react with the fertilizin and considered it to be involved in establishing the block to polyspermy and initiating the development of the egg. As noted above, such an antifertilizin can be extracted from sea-urchin eggs. For this purpose, it is essential first to remove the gelatinous coat of the egg. Upon freezing and thawing of the naked eggs the antifertilizin is extractable by means of sea-water or salt solutions. This substance, too, is of protein nature, isoelectric at about pH 4.5 and inactivated rather readily by heating (e.g., in ½ minute at 75° C. at pH 7). The antifertilizin from eggs shows the same types of action as does the substance from

sperm. Thus, it neutralizes the sperm-agglutinating action of fertilizin, agglutinates suspensions of eggs, and causes the formation of precipitation membrane on the surface of the gelatinous coat of the egg. It also resembles the antifertilizin from sperm in the kind of antibody that is formed when it is injected into rabbits. This antibody is found to agglutinate sperm but not intact eggs. Its failure to agglutinate the eggs is understandable on the basis of the subsurface location of the antigen in the egg. But its action on sperm constitutes further evidence of the antigenic similarity of the antifertilizin from eggs and that from sperm. We have not, as yet, any specific evidence on which to form an opinion as to the role of this material in the fertilization process. However, the finding of such a substance below the surface of the egg presents a situation that seems to me to be of rather general significance. It can, as noted above, combine in serological manner with the fertilizin which constitutes the surface coat of the egg. It appears, then, that a complementary, antigen-antibody like, pair of substances is present in one and the same cell. This leads to the presumption that there may be others present and to the view (77, 79) that such systems of complementary substances form the basis of cell structure.

Complementary Substances in Other Kinds of Cells

I have since made many attempts to obtain such complementary substances from various other kinds of cells and have been able to extract auto-agglutinins from blood cells of mammals and from bacteria, although not consistently. One of the difficulties in such attempts is the probable occurrence of interaction and precipitation of the complementary substances in a process of extraction that involved destruction of the cell. Thus, for example, if surface substance were present in excess, then upon destruction of the cell, it would combine with all of the subsurface substance and the latter would not be obtained in the ordinary extracts. Also, since they may combine in multiple proportions, as antigen and antibody are known to do, and since some may be "univalent" (see 81, 90) the chances of obtaining them readily in the extracts is still further reduced. The ability to demonstrate such antigen-antibody like systems in a cell would depend upon use of appropriate extraction methods and upon the particular properties and amounts of the substances involved. In the case of the sea-urchins we are rather fortunate in being able to remove the gelatinous coat readily without destruction of the rest of the cell.

However, the amount of antifertilizin that we obtain corresponds to approximately one-tenth the amount of fertilizin normally present.

An Auto-Antivenin

One of the properties exhibited by immune antibodies produced against pathogenic organisms, or certain antigens derived therefrom, is their ability to act as protective agents. On the basis of the view I have presented, pathogenic organisms would contain their own antibodies, and it would, therefore, be of considerable interest to learn whether or not protection against the effects of a pathogenic organism or toxin produced by it could be obtained by means of auto-antibodies derived directly by extraction of the organism itself. I have made a number of attempts to obtain such protective auto-antibodies from pneumococci but these have not, as yet, been definitely successful. In view of the various possible extraction difficulties, mentioned above, the lack of immediate success does not constitute a sufficient basis for abandonment of such attempts. In the meantime, more favorable results have been obtained (83) with a different kind of material, namely a venomous reptile called the Gila monster. This animal secretes a venom which is highly toxic to mammals, being lethal to mice in doses of about 0.002 ml. On the basis of the natural auto-antibody concept, a neutralizing agent would be expected to be present in the cells where the venom is formed. Assuming that the venom is synthesized in the venom glands, attempts were made to obtain auto-antivenin by extraction of "de-venomated" glands, but the results were negative. However, it has not been shown that venom is synthesized in the venom glands. So, on the supposition that it may be produced elsewhere and simply stored for secretion in the glands, the search for auto-antivenin turned to serum and to extracts of various other organs. The results showed that serum and an extract of liver were effective. Complete protection of mice against 8 MLD of venom could be obtained with 0.125 ml. of serum, while ten to twenty times that amount of serum of the rabbit or of a non-venomous lizard, the chuckwalla, afforded no protection. The protective agent in the serum was found to be in the globulin fraction.

Our present interpretation of the results is as follows: Venom and antivenin, in combination, are both assumed to be liberated into the blood stream from the organ, probably the liver, in which they are formed. The venom gland then effects a separation of the two

so that venom accumulates in the gland from which it is later secreted while anti-venin is left in the blood. The results, then, do not constitute direct evidence for the auto-antibody concept as formulated above, since it would be essential for that purpose to know definitely where the synthesis occurs. They are, however, favorable to that view, since the expectation of finding an anti-venin somewhere in the animal was based on it.

Some Other Evidence of Natural Auto-Antibodies

In addition to the various experiments with eggs and sperm, bacteria, blood cells and venoms, there exist in the literature various earlier as well as more recent investigations that are probably most readily interpretable on the basis of auto-antibodies. A few of these may be briefly listed here. The most relevant, perhaps, is the demonstration by Kidd and Friedewald (36) that normal rabbit serum reacts in antigen-antibody manner with a sedimentable constituent obtained in extracts of various organs of the same animal. On the basis of the present view, this would be interpreted as due to the presence in the blood of surface substances of the cells of various tissues and that these substances bear configurations complementary to those of the subsurface sedimentable constituents. In fact, one would expect to find complementary substances for all of the macromolecular components of serum located in the tissues where such components are formed. Thus, if the lymphocytes are actually the source of gamma-globulin, as recent evidence (9, 18, 35) tends to show, we should expect to find natural anti-globulin therein. Some evidence has also been presented recently by Rosenow (63) for the extraction of auto-agglutinins from various micro-organisms as a result of prolonged autoclaving in the presence of peroxide. However, since they are found to be volatile, recoverable in distillates, they may represent a different category of substance from that under consideration here. Auto-antibodies which are considered to result from an immunization process have been frequently described in immunological literature. The most well known is the Wassermann reagin found in syphilitic serum, but similar examples are known in cases of other diseases, such as infectious mononucleosis, yellow fever, acute hepatitis, malaria and atypical (virus) pneumonia—(cf. 8, 25, 39, 60, 72, 73, 92). The usual interpretations offered for the origin of such auto-antibodies assume either (a) that the pathogenic organism furnishes a foreign

protein that serves to form a complete antigen with some constituent of the host tissue, or (b) that the pathogen possesses an antigen that is serologically similar to some constituent of the host tissue. On the basis of the natural auto-antibody concept a third interpretation may be offered; namely, that the infectious agent releases, from the tissue undergoing destruction, pairs of complementary substances of which one is quickly removed from the circulation while the other remains there for some time. Thus, in syphilis, for example, it would be assumed that complementary tissue lipoid and protein are released from the infected tissue and that the lipoid, because of its smaller size and other properties, is more rapidly removed from the blood, leaving the complementary protein present in excess. Addition of tissue lipoid to a sample of such blood *in vitro* would then result in the interaction with the complementary protein that is evidenced by the Wassermann reaction.

There are several other cases that may be cited as examples of natural auto-antibodies. Thus, it has been reported (62) that blood of many individuals (about ¼ of all tested) is able to combine with insulin and thereby fix alexin. Other natural anti-hormones and anti-enzymes (cf. 33, 55, 68) may be interpretable as auto-antibodies Certain antibacterial proteins, such as lysozyme (49-52) and diplococcin (56) are derivable from the same species of organism on which they act. In fact, the suggestion has been made by Meyer (49) that "such enzymes which in high concentration partly or completely lyse the organisms from which they are derived are probably concerned with bacterial multiplication", and he (50) pictures these as operating by softening the surface membrane of the cell prior to division. Other autolytic enzymes, such as that of the pneumococcus (2, 7) may belong to the same category. Similarly bacteriophage may be regarded as an example of an auto-antibody, and it has been shown (17, 40) that in certain cases soluble fractions extracted from the susceptible bacterial cells inhibit specifically the lysis of the homologous organisms by bacteriophage.

Growth and Antibody-Formation as Analogous Processes

The view that cells are made up of constituents that bear the same sort of relation to one another as do antigen and antibody leads to the inference that their origin is the result of the operation of the same kind of processes as are involved in antibody formation. For

the formation of immune antibodies there is now fairly general acceptance of the views of Breinl and Haurowitz (4), Alexander (1), and Mudd (54) which have been extended and experimentally supported by Pauling (57, 58). These views involve the incorporation of antigen in the site of synthesis of serum globulin so that, under an orienting influence of the antigen, the new globulin that is formed bears regional structural configurations that are complementary to specific regions of the antigen. We add to this the inference that, in the absence of foreign, introduced, antigen, the normal globulin is complementary to structures normally present at the site of synthesis. Similarly, then, any of the macromolecular constituents synthesized in a cell would be complementary to the substances comprising the sites of synthesis. Since growth consists primarily in the formation of such substances that comprise the integral structure of the cell, we may regard the mechanism of the process of growth to be essentially analogous to that manifested in antibody formation. For the increase of self-duplicating bodies such as genes, one may invoke the formation of substances that are both complementary and identical, as Pauling and Delbrück (59) have done, or the formation of an intermediate template which Emerson (11) lists as an alternative possibility.

Differentiation and Natural Auto-Antibodies

A scheme for differentiation from this point of view involves even more speculation. At present, we cannot do much more than suggest that the antigen-antibody type of reaction is involved. Experimental zoology has provided us with innumerable examples of specific interactions of tissue-cells. By way of illustration two striking examples may be cited. One is the formation of a new sponge from dissociated cells discovered by H. V. Wilson and studied extensively by him (95-98) and by Galtsoff (14-16). Cells of the sponge which have been separated by being forced through fine bolting cloth, re-unite and give rise to a new sponge. This reunion is highly specific since, in mixtures, cells of one species do not coalesce with those of another. The reunion may be regarded as an agglutination phenomenon, and it has, in fact, been suggested by L. Loeb (43, 44) that specific agglutination is the factor which makes isolated cells join into a tissue; that is, that tissues are primarily aggregates of agglutinated cells. In the reconstitution of the sponge, it should be noted that

not all of the various types of cells originally present re-assemble in their proper location. The evidence (98) shows that it is mainly the relatively undifferentiated archeocytes that are involved but that other cells, such as collar cells, that are on hand may be incorporated. This example illustrates the specificity of adhesion of cells, and many other similar cases might be cited. F. O. Schmitt (69) has presented before a previous symposium a surface interaction concept for such adhesion, using the agglutinating action of histone on erythrocytes as a basis. It should be noted, however, that it is not necessary to postulate a basic protein for this purpose. It would suffice to assume the presence of a film of protein or other macromolecular substance having a structure complementary to the subsurface material. Upon dissociation of the tissue rupture of the film on parts of, or all of the surface of, some of the cells would leave exposed subsurface substance which could then combine with the intact film of other cells. A similar concept may be applied to the adhesion of cells of different tissues and Weiss (94) has, in a previous symposium, diagrammed various possible arrangements of complementary proteins that would account for such tissue affinity as occurs, for example, in the union of outgrowing nerve fibers with the cells of their specific end organs.

The second set of examples is derived from experiments by Holtfreter (29-32) on the behavior of mixtures of tissues. He has shown, in the first place, that isolated cells and clumps of cells of like, as well as unlike, prospective significance will fuse. Fusions of like cells persist, but certain combinations of cells of unlike prospective fate later separate. However, such separation, as of ectodermal from endodermal cells may be prevented by the interposition of mesodermal material. These interactions occur only as a result of contact and do not involve an action at a distance. Holtfreter (32) has shown, too, in studies of the mechanics of gastrulation that any embryonic tissue, provided it is partially deprived of its surface coat, will become engulfed by an edodermal substratum, but only in homologous combinations will they interlock. The specific forces involved in these fusions of tissues are again interpretable as due to the action of complementary antigen-antibody like substances.

While the extent of complementariness of surface structures may serve as a basis for accounting for the specific adhesion or non-adhesion of cells and tissues, and their resulting organization into organ systems, we must also inquire as to whether they may be

involved in the actual differentiation of the cells. An indication that such may be the case exists in work on the chemistry of the so-called organizer. Holtfreter (27, 28) showed that the protein residues after ether extraction were much better neural inductors than any of the extracts and Barth and Graff (3) have succeeded in obtaining good neural tube induction by means of protein extracts of active tissue. Inductive action appears, then, to be associated with such macromolecular material, and the presence of a specific type of protein, or other macromolecular substance, on the surface of the invaginating chorda-mesoderm, rather than any special metabolic properties of this material may be responsible for the normal induction of neural tissue. In this connection reference should also be made to a report of some experiments by Weiss (93) in which antisera produced against autolyzed suspensions of adult organs were injected into the developing eggs. He found that, while there were general depressive effects on the growth of liver and kidney, these organs showed statistically significant increase in those cases where antisera specific for the organs were employed. He suggests, too, that if similar reactions take place in the organism, this would supply a biochemical growth-controlling mechanism of great specificity and selectivity.

We know from the extensive work on the antigens of the blood cells that the specific structure of the molecular constituents of the cell surface is gene determined. It appears particularly from the work on doves (reviewed recently by Irwin, 34) that the antigens are rather direct products of their causative genes, each antigen being produced by a specific gene with no influence, in general, of other genes or of the environment. Since all of the cells of the organism are supposed to contain the full complement of genes one might then expect to find the same sets of antigens present on the surfaces of all of the cells. R. S. Cooper's (5) finding of adult antigens in the egg and embryo of the frog may be an expression of this. However, while certain antigens such as the A and B structures of humans are found on most other cells and tissue fluids, other antigens, such as M and N, are restricted to the blood cells. This situation may be accounted for in various ways. One might simply assume that all of the genes are active in producing antigens but at different rates in different tissues so that certain of the antigens do not reach the surface of the cell. We may, then, conceive of differentiation as involving differences in the rates of production of different antigens, at first

in different regions of the uncleaved egg, later in different blastomeres and embryonic tissues. If production of these substances proceeds in the antigen-antibody manner that we have suggested, then we may expect that a particular surface substance on one kind of cell may be represented by the complementary substance on another type of cell. This is the situation in the case of eggs and sperm and it seems reasonable to suppose that cells of other tissues of the organism may bear such complementary structures to a greater or lesser degree. Thus, if in two kinds of cells surface material represents mainly products of one particular gene, one cell may bear a particular antigen and the other its complementary substance. From the work on blood cells of mammals we know, however, that a large number of different antigens may be present on the surface, and we can surmise that the same may be true for cells of other tissues. The relative affinity of different tissues would depend, then, on the extent to which these antigens are represented by the complementary substance in the different tissues.

The work on the specific substances of eggs and sperm also gives some indication of the presence of substances of like specificity inside the cell in one case and on the surface of the cell in the other. Thus the antifertilizin from the inside of the egg resembles that on the surface of the sperm in reacting specifically with the coat of the egg. Immune antibodies prepared in rabbits against these two substances are found to agglutinate and be absorbed by sperm but not react with the intact eggs. Thus the antifertilizin from within the egg is serologically equivalent to that on the surface of the sperm. There are various other examples, that might be cited, of the presence of the same specific substance on the surface in one kind of cell and below the surface in another kind. Perhaps the best illustration is the difference between smooth and rough strains in micro-organisms such as the pneumococcus. There the protein antigen of the surface of the rough variant is a subsurface substance coated over by the specific polysaccharide in the smooth form. This kind of situation may also be involved in the experiments on the "dead organizer". Holtfreter (27, 28) showed that tissues which lacked inductive capacity while alive could acquire such activity when killed in various ways. It is quite conceivable that the non-inducing living tissue contains the same specific inductor as does the dorsal-lip of the blastopore but that it is located in a subsurface position from which it is released by appropriate destruction of the cell.

The general view presented here postulates that all of the cells contain all the diverse specific antigens of the organism, but in widely different amounts and in different locations in the different tissues. To account for these differences in location and amount we must again fall back on initial regional differences in the cytoplasm of the uncleaved egg. One specific scheme would involve the inactivation of various genes by interaction with complementary substances in different parts of the cytoplasm. This would be analogous to the proposals of Sturtevant (71) and Emerson (10) for the induction of mutations by means of antisera. Many kinds of schemes might be formulated but the present state of our information hardly justifies extended speculation along this line. It is obviously essential for us first to obtain further evidence for or against the existence of such antigen-antibody like systems of complementary substances within cells.

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PLANT TISSUE CULTURE

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MR. PRESIDENT, LADIES AND GENTLEMEN: It is a great honor to be asked to address such a select company of American Biologists. It gives me great pleasure to be in this most progressive of the world's countries. I owe this pleasure primarily to Dr. White: he took the initiative to have me join you. I want to thank him very deeply. I want to express also my gratitude to the organizing committee of this symposium, who have had the kindness to invite me.

Before entering upon the subject of my lecture, I want to express my country's gratitude to the American nation. After a hopeless struggle we were overwhelmed by the powerful, numerous and well-equipped forces of an enemy. We had to face the cruelty of defeat, and the bitterness of oppression. It was only the hope of help from abroad which sustained us during the dark years, and from the first day, we put our confidence in the power of the United States of America. When, after four years of expectation, the Allied Armies at last trod the soil of our country, an immense enthusiasm surged through the people of France. We understood that the time had come for our liberation. We felt for you a gratitude nothing can blot out.

It was your intervention which drew us out of the depths where defeat had plunged us. This, France has not forgotten.

Ladies and Gentlemen, the lecture I am to deliver here concerns plant tissue culture.

Ever since Haberlandt defined this problem in 1902, many researchers have tried to solve it. But for a long time their attempts remained unsuccessful, because they were made upon differentiated tissues, composed of cells which had lost the power of multiplying. Only after twenty years of failure was the way to success made clear. Kotte and Robbins opened it simultaneously. These authors were the first to understand that it would be possible to obtain tissue cultures only if the cells used were cells capable of normal proliferation, that is meristematic cells. They started from root meristems, and thus realized root cultures.

But neither Kotte nor Robbins succeeded in obtaining unlimited growth. Nine years passed, during which the solution of the problem of plant tissue culture seemed still far away. But with 1931, a conclusive period started. White, resuming Robbins' researches, after striving for three years, succeeded in obtaining unlimited growth of excised roots. At the same time when your compatriot announced this fundamental result, that is in 1934, I had begun my own researches, which had resulted in the culture of the cambium of certain trees, for a limited period of a few months. At that time, the problem of tissue culture was obviously not fully solved: the unlimited cultures realized by White were in fact cultures of organs, with defined form and structure, and it could not be hoped that real tissue cultures of tissue culture was obviously not fully solved; the unlimited cultures were no complete answer either, for even though they were real tissue cultures, they were unfortunately of limited life. The two conditions of tissue culture, that is unlimited growth and lack of organization, were realized, but separately. But, encouraged by such results as White's and my own, researches were resumed, and some years later resulted in complete success. Realization of tissue cultures capable of unlimited growth was announced by White, on December 30, 1938, by myself on January 9, 1939, and by Nobécourt on February 20, 1939.

The world war started a few months after the publication of these results. Being unable to communicate with one another, White and I have for five years conducted our researches in quite different directions.

White tried to apply plant tissue culture methods to the study of pathological problems. His first experiments had already given a hint of this, as the Tobacco tissues he grew came from a sort of neoplasm, which could be called a genetic tumor.

It was natural that White should try to extend this work to different tumors, particularly to those produced by *Phytomonas tumefaciens*. You are all aware of the remarkable results White has obtained along this line; I will not discuss them here. I will only recall that, by means of irreproachable experiments, your compatriot showed that, even after being completely cleared of the specific bacteria, Crown-Gall tissues grow faster and longer than corresponding normal tissues. The discovery of this remarkable fact permitted him to affirm the existence of true cancers in plants. After briefly recalling the re-

searches realized in America, I shall now tell you what results were obtained in my own laboratory, which have made possible the realization of tissue cultures of many species.

I shall first consider the development of isolated fragments of normal plants, that is, the first part of tissue cultures. Then, I shall survey the methods of transplantation of the cultures, and the action of heteroauxins upon their growth. Finally, I shall indicate some applications of tissue culture in plant Biology.

Let us consider, to begin with, the first stage of tissue culture. To obtain it, it is often sufficient to place an organ in a medium containing simply the mineral salts of Knop's solution, and glucose. If, for instance a fragment of cambial tissue of *Acer pseudoplatanus* is grown in such conditions, it produces, in places, small parenchymatous lumps. In the case of the cambial tissue of *Salix capraea*, the proliferation is more important. The fragment produces, at one extremity, a voluminous parenchymatous mass. Those parts which are plunged into the nutrient do not show any visible development. This is a demonstration of the inhibiting action of contact with an aqueous medium upon the growth of the cambial tissue of a tree.

After thus examining superficially the tissues, let us now study their structure. When examined under the microscope, with reflected light, the surface of a cambial tissue fragment grown *in vitro* shows round, very turgescent cells, which seem almost isolated from one another, and form over the whole, a sort of delicate down.

It is possible to excise a part of this down, and to examine it under the microscope by transmitted light. It can thus be seen to consist of pluricellular rows often branched, and similar to certain fungus thalli. We have called these curious formations pseudothalli; they have been known for a long time, as they appear during healing processes. To study the structure of deep regions of the excised parts, histological sections are necessary. The examination of such sections shows that the voluminous formations which grow in regions isolated from the medium have a structure different from that of normal tissues. The pre-existing tissue consists of a phloem fragment, one side of which is lined with the true cambial tissue. The cells of this pre-existing tissue are regularly set in radial lines. The newly-formed tissue on the contrary, consists of a sort of parenchyma, where the cells are placed with no particular arrangement. Sometimes this parenchyma contains in places outlines of cambium initial layers giving, on one

side, a sort of phloem, and on the other, clusters of woody cells, which can be looked upon as aberrant vessels.

Until now, we have considered solely the growth of tissues from woody species. But these are not the only ones we know how to grow. Growth *in vitro* can be obtained also with fragments of herbaceous stems, such as Tobacco or *Antirrhinum,* or rhizomes such as that of Jerusalem artichoke, or root tubers, such as Carrot, Chicory, *Scorzonera,* and so on.

If for example a slice of Carrot is grown *in vitro* it produces a voluminous, ring-shaped callus in its central part. This callus is very thin in the center of the Carrot slice and gets progressively thicker towards the edge of the slice. The maximum thickness occurs even with the cambium initial layer. On the other hand, the regions situated just outside the initial layer, that is the phloem regions, divide only slightly. (Fig. 1).

Thus the newly-formed tissues in a Carrot slice are produced by the cambium initial layer, and by the youngest regions of the vascular parenchyma. It is remarkable that the newly formed tissues are located exclusively on one surface of the slice. This is proof of the tissues' polarity, which is remarkably distinct in Carrots.

The sprouting side is always the lower surface of the slice, that is that which was turned towards the root tip in the intact organ.

To try to analyze the exact growing process of the callus produced upon a carrot slice, it is necessary to examine histological sections through the newly-formed tissue and the pre-existing elements. By this means, we have observed that at first the slice produces a homogeneous parenchyma-like tissue; later the cells lying at the limit between the pre-existing tissue and this newly-formed parenchyma grow actively and give rise to a row of flat cells which form a perfectly distinct cambium initial layer.

Finally, the initial layer functions actively, producing outwardly a parenchyma in which sieve tubes are visible and inwardly a vascular parenchyma where woody cells are differentiated which can be looked upon as vessels.

When the callus is thus formed it consists of, starting from the outside (Fig. 1): the parenchyma formed in the early stages of the culture (P), some phloem (PN), a criblo-vascular zone (CN), some vascular parenchyma (VN), and finally the pre-existing tissue.

Such a disposition is quite similar to that of normal organs: If

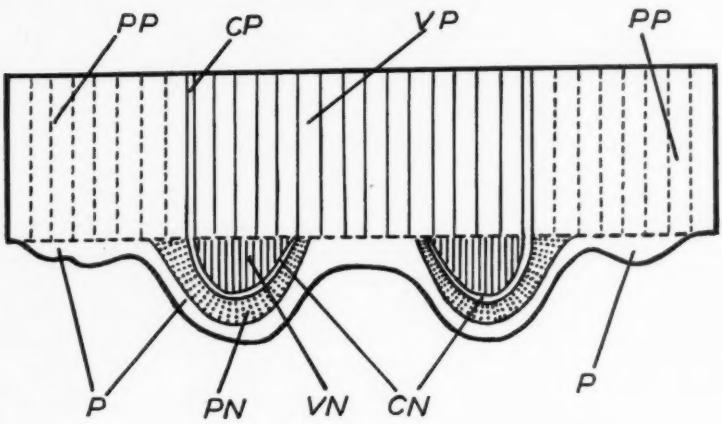
sections are made in a stem with secondary formations, they show, starting from the outside: a cortical parenchyma, phloem, the cambium, vascular parenchyma or vessels, and in the center, medulla. We have a right to say, accordingly, that during its growth *in vitro*, a carrot slice tends to reproduce a normal structure.

In the course of my researches, I have been able to ascertain a great diversity in the behavior of fleshy organs from many species, when grown *in ivtro*. I shall not describe all the different types of growth represented in tissue cultures of fleshy organs. I shall present only the example of carrot cultures.

To complete this survey of the first stages of tissue cultures, let us consider now the case of lianes, upon which successful experiments have been made. This material has mainly been studied by my collaborator, Mr. Morel. He has succeeded in growing tissues from many lianes, such as Grape, Virginia-creeper, Bramble, and so on. Figure 2 represents a stem fragment of Grape, which has produced a voluminous callus.

After thus considering the first stage of tissue culture, that is the development of excised fragments of normal plants, let us now study the transplantation of cultures. To make a transplant from a primary culture, a callus fragment chosen in the most actively multiplying region is cut and transferred to a new medium. This fragment grows in all directions, and is slowly transformed into a rounded mass. One or two months later, depending upon the species, another transplantation is necessary, and so on. It has been possible, in this way, to separate tissue strains from many species, and to maintain their development indefinitely. You may recall that, in this country, White has been keeping Tobacco tissues, Sunflower Crown-Gall tissues, etc. . . . for many years. In my laboratory, strains from sixteen species have been grown for periods of two to nine years. Figure 3 represents the appearance of such a culture. This is a colony of Carrot tissue coming from a strain aged six and a half years after thirty-nine transplantations. The colony, which is 45 days old, is rounded and presents many round lumps. Figure 4 represents a Grape tissue culture, from a strain aged eighteen months and transplanted nine times. As in the case of the Carrot cultures, many rounded lumps can be observed.

We have found that plant tissues, contrary to the behavior of animal tissues, can grow for a long time without being transplanted,



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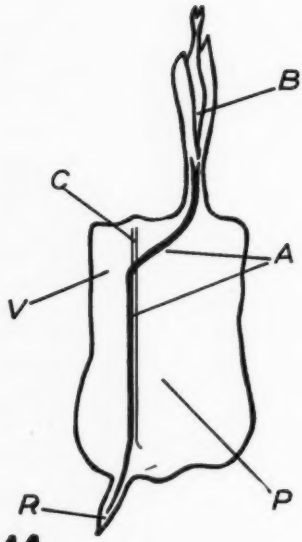

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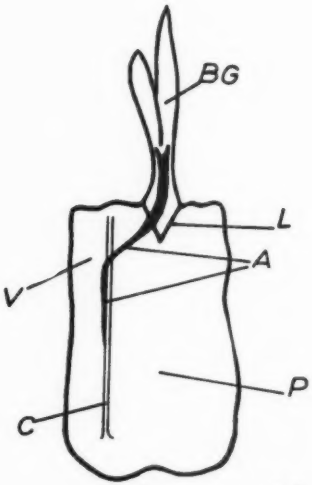

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FIGURE 1

RADIAL SECTION OF A CARROT SLICE GROWN *in vitro*

The lower surface of the slice has produced a voluminous callus. This callus is very thin in the center of the slice and becomes thicker towards the edge. The maximum thickness occurs at the initial cambium layer (CP). This callus consists of, starting from the outside, parenchyma (P), phloem (PN), newly formed cambium (CN), vascular parenchyma (VN) and finally the pre-existing tissue, PP—pre-existing phloem, VP—pre-existing vascular parenchyma.

FIGURE 14

HISTOGENIC ACTION OF A BUD DEVELOPED ON THE PHLOEM OF A FRAGMENT OF CHICORY ROOT

B, bud; C, cambium; P, phloem; V, vascular parenchyma; R, root; A, alterations induced in the pre-existing tissue by the presence of the bud.

FIGURE 15

HISTOGENIC ACTION OF A BUD GRAFTED ON THE PHLOEM REGION OF A PIECE OF CHICORY ROOT

BG, grafted fragment with a bud; L, limit between the scion and the stock; P, phloem; V, vascular parenchyma; C, cambium; A, alterations induced in the stock by the grafted bud.

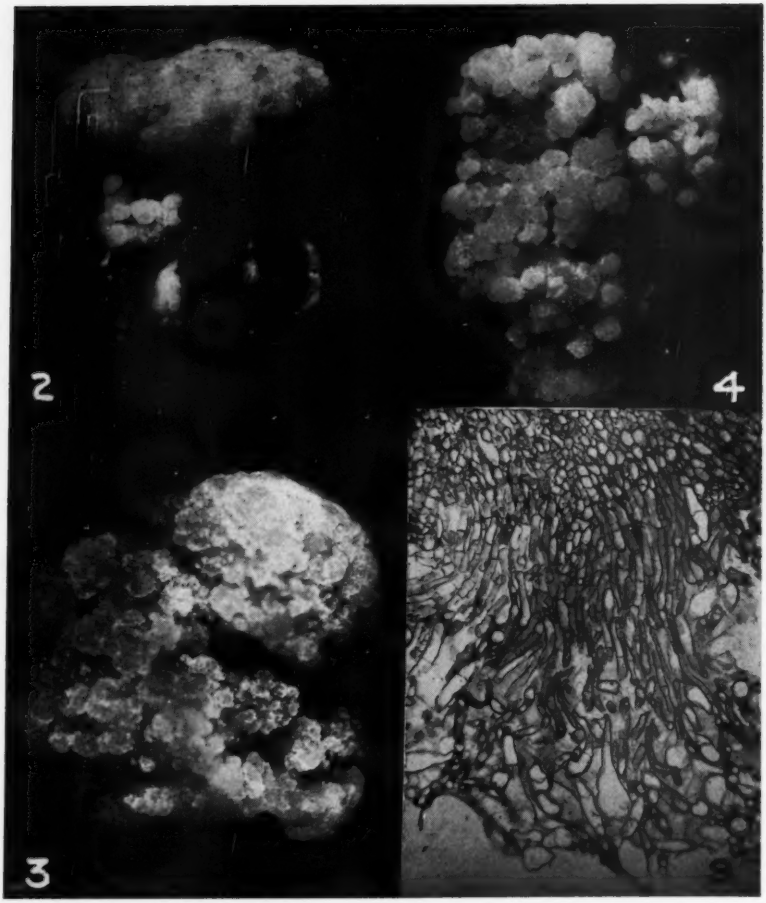

FIGURE 2
STEM FRAGMENT OF GRAPE GROWN *in vitro* FOR ABOUT TWO MONTHS
It has produced a voluminous callus.

FIGURE 3
COLONY OF CARROT TISSUE, AGED 45 DAYS, COMING FROM A STRAIN SIX AND A HALF YEARS OLD (39TH TRANSFER)

FIGURE 4
COLONY OF GRAPE TISSUE AGED 2 MONTHS COMING FROM A STRAIN 18 MONTHS OLD (9TH TRANSFER)

FIGURE 9
ANATOMICAL STRUCTURE OF A JERUSALEM ARTICHOKE FRAGMENT GROWN IN A NUTRIENT CONTAINING INDOLE-ACETIC ACID AT A CONCENTRATION OF 10^{-5}
The cells have swelled in all directions (isodiametric enlargement).

provided that a large enough container be used. Thus we have obtained giant colonies. One of them, after ten months without transplantation, was almost twelve centimeters in diameter and weighed over 150 grams.

After thus studying the morphology of transplanted tissues, let us now examine their structure. It is different from that of normal tissues. In most cases, the colonies have a heterogeneous character and consist mainly of a parenchymatous mass, in which clusters of small cells represent vascular bundles. Sometimes these formations are similar to normal vessels. But more often they are irregular and consist of a group of woody cells of various forms together with a cambium and an anomalous phloem.

Finally, in certain cases, the cribro-vascular structures do not differentiate but remain in the shape of a cambium consisting of irregular nodules.

Such cultures can be considered as constituted of cambial tissue and its derivatives. This frequent type occurs in tissue cultures of Carrot, Chicory, *Scorzonera,* Virginia-Creeper, Bramble, and so on.

But it is not universal. In the case of the Grape tissue, for example, Morel has obtained a homogeneous strain which is wholly parenchymatous and shows no secondary differentiation. Thus not only is it possible to obtain cultures of cambium and its derivative tissues, but also pure parenchyma cultures. In my laboratory have been obtained tissue strains of the following species: *Daucus carota, Brassica campestris, Helianthus tuberosus, Cichorium Intybus, Scorzonera Hispanica, Hyoscyamus niger, Vitis vinifera* var. Aramon, *Vitis Coignetiae, Vitis Davidii, Vitis incisa, Parthenocissus tricuspidata, Parthenocissus Hederaefolia, Cissus discolor, Rubus fructicosus, Antirrhinum majus, Crategus monogyna.* The growth of those strains is excellent and we can assert that it will be indefinite. We have strains of other normal tissues such as *Helianthus annuus, Nicotiana tabacum, Salix capraea, Althaea malva;* but their development is slow and we do not know if it will be indefinite.

The aspect of the colonies varies from one species to another and is quite characteristic. On the other hand, for single species, the aspect of the colony depends on its structure. For instance, Morel has two kinds of Grape strains. The first, consisting of a parenchyma associated with cambial initial layers, phloem and woody cells is characterized by compact and opaque colonies. The other strain, con-

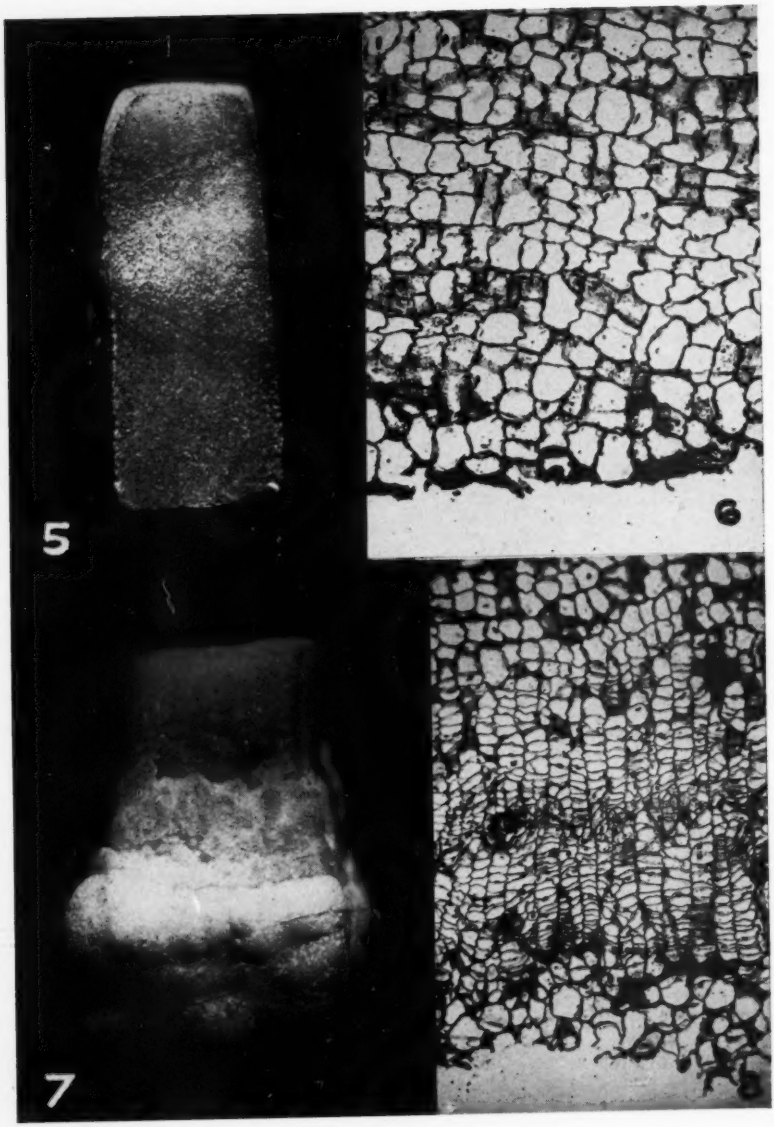
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FIGURE 5

FRAGMENT OF A JERUSALEM ARTICHOKE TUBER GROWN IN A NUTRIENT DEPRIVED OF INDOLE ACETIC ACID

This fragment has not developed.

FIGURE 6

ANATOMICAL STRUCTURE OF A JERUSALEM ARTICHOKE FRAGMENT GROWN IN A NUTRIENT DEPRIVED OF INDOLE ACETIC ACID

The cells have not divided.

FIGURE 7

FRAGMENT OF A JERUSALEM ARTICHOKE TUBER GROWN IN A NUTRIENT CONTAINING INDOLE-ACETIC ACID AT A CONCENTRATION OF 10^{-7}

The lower part, which was plunged in the medium, has produced a voluminous callus.

FIGURE 8

ANATOMICAL STRUCTURE OF A JERUSALEM ARTICHOKE FRAGMENT GROWN IN A NUTRIENT CONTAINING INDOLE-ACETIC ACID AT A CONCENTRATION OF 10^{-7}

The fragment has produced a cambial zone with vascular structures.

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FIGURE 10

FRAGMENT OF CARROT ROOT GROWN IN A NUTRIENT CONTAINING INDOLE-ACETIC ACID AT A CONCENTRATION OF 10^{-7}

It has produced voluminous callus.

FIGURE 11

PHLOEM FRAGMENT OF CARROT GROWN IN A NUTRIENT CONTAINING INDOLE-ACETIC ACID AT A CONCENTRATION OF 10^{-6}

It has produced numerous roots.

FIGURE 12

SLICE OF CHICORY-ROOT GROWN IN A NUTRIENT CONTAINING NAPHTHYL-ACETIC ACID AT A CONCENTRATION OF 10^{-7}

It has produced many roots.

FIGURE 13

SLICE OF A CHICORY ROOT GROWN IN A NUTRIENT DEPRIVED OF HETERO-AUXIN

It has produced several buds.

sisting only of a parenchyma without any differentiated structures, is characterized by friable and translucent colonies. Thus, there is a connection between the aspect of cultures and their structure.

The speed of growth is variable. For example, the development of *Daucus* or *Parthenocissus* tissues is very rapid whereas the growth in *Hyoscyamus* and *Brassica* tissues is very slow. Nevertheless, the slowness of the growth is not a bar to the possibility of indefinite culture; our strain of *Brassica* tissue, for instance, is now six years old.

Our cultures thus come from numerous enough species to demonstrate that the realization of real tissue cultures is not exceptional. Let us stress, however, the fact that so far only dicotyledonous tissues have given satisfactory results.

I have told you what are the morphological characteristics of plant tissue cultures. I shall now consider their nutrition.

I said, awhile ago, that it is possible to grow isolated fragments of normal plants upon a very simple medium, containing only mineral salts and glucose, hardened with agar; but such a nutrient can be used only during the first stages of culture and does not maintain lasting growth.

In order to obtain indefinite development, a more complex medium must be employed, containing, besides mineral salts and a sugar, various organic substances acting as vitamins. These nutrients which make possible the indefinite growth of cultures, are of two sorts. Those used by White are related in their composition to the nutrient solutions commonly used to grow excised roots. The most commonly used consists of mineral salts, glucose, agar, nicotinic acid, vitamin B_6, glycine and finally vitamin B_1. This nutrient gives excellent results with the tissues of the Tobacco hybrid which White has used in his researches. It is also satisfactory when used to grow Crown-Gall tissues. But its use cannot be extended to all cultures, for it lacks heteroauxin which is, as we shall see, an essential growth factor for many plant tissues. In the course of the researches which I and others have carried on for the past ten years and which are still under way, the action of heteroauxin upon plant organ fragments grown *in vitro* has been analyzed. This study has been very useful in the perfecting of nutrient media for plant tissues. I am going to tell you of these researches, before indicating the composition of the nutrients used in my laboratory. The most demonstrative experiments were made upon tissues such as those of Jerusalem artichoke which grow only in the presence of heteroauxin.

Figure 5 represents, for instance, a tubercle fragment of this species grown in a nutrient deprived of heteroauxin: this fragment displays no sign of development. The examination of an anatomical section (Fig. 6) confirms this conclusion, as it reveals that the cells have not divided.

Figure 7 shows on the contrary a fragment similar to the preceding one but grown on a nutrient containing heteroauxin at a concentration of 10^{-7}: it has produced a voluminous callus in the region in contact with the nutrient. A section shows that the callus consists of a cambium with vascular structures (Fig. 8).

It may be said that, when used at a concentration of 10^{-7}, indole-acetic acid possesses cambium-forming properties.

If the concentration is raised to 10^{-6}, the power of root formation is added to the cambium-forming properties of heteroauxin, as the tissues form not only cambial layers but also root meristems.

If the concentration is further increased, if, for instance, a medium is used with 10^{-5} indole-acetic acid, new reactions can be observed. The acid ceases to promote cell-division. It determines instead the swelling of the cells in all directions, that is to say their isodiametric enlargement.

Figure 9 represents a section upon which this phenomenon is particularly noticeable. At the top are cells which have undergone no change at all. In the lower part of the picture, elements can be seen which have swelled under the influence of heteroauxin.

These three types of action of heteroauxin upon plant tissues are very common and especially evident upon herbaceous plant tissues. We have observed them in many cases; the behavior of the Carrot tissue is particularly clear. Figure 10 represents, for example, a fragment which has undergone the action of a 10^{-7} dose of indole-acetic acid. It has grown a callus much bigger than those normally produced.

Figure 11 shows a fragment which has been grown in the presence of a dose ten times greater, that is 10^{-6}. It has grown many roots.

Finally, when a fragment is grown in a nutrient with a heteroauxin concentration of 10^{-4}, it produces giant cells which emphasize the action of heteroauxin upon the isodiametric enlargement of cells.

To finish with this study of the action of heteroauxin upon the tissues we must consider the inhibiting power of this substance towards bud formation. Figure 12 represents for instance, a slice of Chicory root, grown upon a nutrient containing naphtylacetic acid at a con-

centration of 10^{-7}; it has produced roots and a large callus while another slice grown without heteroauxin (Fig. 13) has mainly produced young shoots.

Thus, the analysis of heteroauxin action upon plant tissues has proved that a concentration of 10^{-7} has a specific action upon cell division. It seemed natural that this power should be used to make better tissue culture growth. So we have prepared nutrients containing indole-acetic acid, or naphtylacetic acid, with concentrations approximating 10^{-7}.

The following table indicates, for example, the composition of the most frequently used among these heteroauxin-containing media:

Water	1000 g
Calcium Nitrate	0.50
Potassium Nitrate	0.125
Magnesium Sulfate	0.125
Monopotassium Phosphate	0.125
Iron, Manganese, Iodine, Chlorine, Cobalt Titanium, Zinc, Copper, Beryllium, Boron, Traces	
Glucose	20 to 50 g
Thiamine	10^{-6}
Cysteine chloride	10^{-5}
Indole-acetic or naphtyl-acetic acids	10^{-8} to 3.10^{-7}
Agar	12 to 15 g

This nutrient contains mineral salts, an organic source of carbon in the form of glucose, agar intended to harden it, and three organic substances of vitamin value, cysteine, thiamine, and a heteroauxin. These various vitamin substances are of varying importance. The first two, that is cysteine and thiamine, have negligible roles, as their suppression results only in an undecided decrease of the growth rate and does not prevent growth from going on indefinitely. Heteroauxin, on the contrary, represents an essential factor for the growth of many tissues. Grape, *Brassica campestris*, var. *naphobrassica*, *Scorzonera*, Carrot, and so on, grow only slightly and slowly in a nutrient deprived of heteroauxin, while their development is at least five times quicker and lasts indefinitely, if indole-acetic acid or naphtyl-acetic acid are used.

Thus, heteroauxin is an essential constituent of nutrients for plant tissue cultures.

Yet repeated experiments have shown that, if certain tissues are subjected for a long time to the action of heteroauxin, they become capable of growing indefinitely without it. I have designated this curious phenomenon with the name of "habituation" to heteroauxin,

for the tissues which present it lose almost completely their reactivity towards heteroauxin. Moreover, they present a series of special characteristics which make it possible to distinguish them from non-habituated tissues. For one thing, they are translucent and friable, consisting of small nodules arranged in a not too coherent fashion, while normal tissues are compact and opaque.

Such differences in the aspect can be observed upon several tissues: *Scorzonera,* Virginia Creeper, Carrot and Grape. I want to point out the fact that tissues habituated to heteroauxin differ also from normal tissues in that they divide more rapidly in whatever medium may be used, and are unable to form roots.[1]

These particular characteristics of tissues which have undergone the prolonged influence of heteroauxin must be compared to the phenomena discovered by White in the course of his researches upon Crown-Gall tissues. Indeed, this scientist observed that cultures obtained from secondary tumor tissues, deprived of the specific bacteria, differ in their characteristics from normal tissue cultures. The tumor tissue colonies are translucent and of a granular texture, while the normal tissue colonies are opaque and dense. Also, the tumor tissues grow rapidly and indefinitely in White's nutrient medium, which is deprived of heteroauxin, while normal tissues grow but slowly, and for a limited time. In the presence of such results, we may suppose that the transformations brought about by heteroauxin's prolonged action are similar to those produced by *Phytomonas tumefaciens,* that is that they represent a sort of cancerisation. The future will tell if this comparison is well-founded.

Thus, while examining the most recent developments of plant tissue culture, we are led to turn towards the future. Many questions are yet unanswered, many problems await our attention. One of the main ones is that of growth-substances. Among the host of compounds so far considered as having the power of stimulating cell division, plant tissue culture helps establish a graded order. It has already proved that heteroauxins are true division substances, while some other factors, such as cysteine, usually considered as growth-stimulant substances, have very slight influence. Quite recent researches, though, lead us to think that in large doses, vitamin B_1 may be a cell division

[1]A culture of the habituated strain of carrot kindly given me by Dr. Gautheret, has produced a root under Philadelphia conditions, as did also two cultures of *Brassica.* (P. R. White).

excitant. Let me point out too, that the work of my collaborator, Morel, has established that pantothenic acid is a very powerful division substance which makes it possible to obtain the unlimited growth of Hawthorn tissues.[2] But these researches are just at a start and must be completed. I must also mention such substances as traumatic acid, which is used in this country, and which has not yet been submitted to the severe control of plant tissue cultures so that its exact value as a growth substance is not yet well known. Let us hope that this gap will soon be filled and that also all the other substances which are thought to be cell division excitants will be experimented upon in tissue cultures.

Another future problem for plant tissue culture will consist in extending this technique to plants widely different from those grown so far. Researches should, so it seems, be tried upon Monocotyledons or Gymnosperms which have been neglected up to now. Higher Algae and Pteridophyts might also be material for new attempts. Finally, I think that the applications of Plant Tissue Culture in Plant Pathology must be developed. White was the pioneer in this direction a long time ago, in growing viruses along with excised Tomato roots. Later on, he took up the problem of Plant Cancer, and you are aware of his success.

In France, my collaborator Morel has used plant tissue cultures to maintain the development of obligate parasites. He turned first to the culture of Grape mildew. In a first set of experiments, he succeeded in obtaining the development of this fungus on the surface of the calluses produced by Grape stem fragments grown *in vitro*. The mycelium of the mildew grew on the surface of the callus and also in the intercellular spaces of the parenchymatous tissue of the callus. It is capable of producing many conidia. In this method, the mycelium grows very rapidly, kills the Grape fragment in a few weeks, and then loses the power of developing. Frequent transplantations to healthy calluses are therefore necessary, which is impractical, and necessitates a great quantity of aseptic material. This method is then quite imperfect. As a remedy, Morel has grown mildew upon a strain of tissue cultures two years old, and already transplanted twelve times. Here, the fungus growth is much feebler and the tissue colony is invaded but slowly. It is then possible to transplant at the same time both Vine tissue and Fungus, which makes the operation much simpler.

[2]Recently, Morel has observed that Hawthorn tissues require Biotin.

Morel has grown such associated cultures for twelve months. This realization of Mildew cultures is extremely valuable in that it enables the researcher to work at any time instead of being dependent on natural and seldom regular circumstances for this observation.

Guided by similar principles, Segrétain and Morel have succeeded in growing Tobacco-mosaic virus on tissue cultures of *Nicotiana tobacum*.

These first experiments of White and Morel show the great interest of tissue culture for pathologists.

Finally, I shall mention the applications which this technique should find in the field of morphogenesis. I want to point out first that White and myself have used it to explain the determinism of budding. I used it, too, to analyze the phenomena of plant polarity. Quite recently, my collaborator, Camus, made use of this method to demonstrate the histogenetic action exerted by buds upon subjacent tissues. Let me indicate some details about these very original researches.

If a root fragment of Chicory is grown, buds can be seen to appear in the region of the cambium and phloem, on the upper surface of the slice (the region which turned towards the stem before the fragment was excised). Observing histological sections, Camus verified that the tissues placed immediately under the buds undergo quite characteristic alterations (Fig. 14). These alterations begin with the multiplication of the rows of small cells which cross the phloem parenchyma radially. Later, inside the meristems, in the lumps thus formed, cribro-vascular bundles differentiate, which are continuous with the bundles of the buds. These structures plunge obliquely into the tissues and are connected with the cambium of the root fragment. Under the junction, the cambium produces numerous vessels connected with one or two roots which are formed on the lower part of the fragment.

Camus has made similar observations upon buds formed on the lower surface of a Chicory root fragment, that is in the region which was turned towards the root tip in the intact organ. But in that case, he has not observed any transformations in the subjacent tissues. Therefore, he believes that buds have a histogenetic action which is transmitted in the tissues in a leaf-to-root direction.

In order to obtain still more conclusive results, Camus has repeated the experiments by grafting a bud on a tissue fragment. In this way, he observed that the histogenic action of the bud is capable of crossing the line of fusion and of appearing in the supporting tissue, as was the case in the preceding experiment (Fig. 15).

We are, of course, permitted to suppose that this action produced by buds in subjacent tissues is due to the secretion of a hormone. The future will tell if we are right.

The few examples I have just recalled will show, I hope, that plant tissue culture has a wide scope. This method may be applied to many other problems. It will facilitate a greater knowledge of plant cell nutrition and respiration; it will also make it easier for new and original data on the determinism of structures to be formulated. In this direction it has already enabled us to establish certain facts regarding the determinism of cambium.

To sum up, studies carried on for the past 15 years or so have already resulted in important conclusions. Unlimited growth of tissues from some 16 species from the most various groups of Dicotyledons have been realized. These researches have initiated a serious study of growth substances and their action upon plant tissues. Among these substances, one, heteroauxin, has the astonishing power of progressively changing tissues' properties, and of making them become similar to Crown-Gall tissues.

Tissue culture, finally, has enabled scientists to study a great many interesting problems. With its help, White has demonstrated the reality of plant cancer, Morel has succeeded in growing Mildew. Working upon organ fragments grown *in vitro,* Camus has demonstrated that buds determine organization phenomena in subjacent tissues. I have myself employed this method to analyze plant polarity and to ascertain the determinism of some anatomical structures.

All the questions I have briefly mentioned represent but a small part of the problems which plant tissue culture may help to solve. This new method opens unlimited possibilities and we can look towards its future with confidence.

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CERTAIN PHASES OF THE APPLICABILITY OF LONG TERM TISSUE CULTURES IN A STUDY OF GROWTH AND DIFFERENTIATION*

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This is a summary of certain data obtained from a series of studies which have been in progress at the National Cancer Institute, Bethesda, Md., on the action of one chemical carcinogen, 20-methylcholanthrene, in inducing changes in a strain of mouse subcutaneous connective tissue fibroblasts growing in long term tissue culture in an entirely heterologous culture medium. In this presentation the author chose to consider the malignant cell not as a de-differentiated cell but as a cell which has undergone an aberrant differentiation. Altered morphology, altered rate of proliferation both in vivo and in vitro and other obvious manifestations of altered physiology were considered, irrespective of their clinical significance, as indices of such aberrant differentiation.

The cultures discussed in this study all arose from a small clump of subcutaneous connective and adipose tissue taken from a one hundred day old C3H strain mouse. The cultures were all grown in Carrel D 3.5 flasks in an entirely heterologous culture medium which consisted of chicken plasma, horse serum, chick embryo extract and an isotonic saline solution. To date this study has been carried over an interval of nearly six years. The behavior of six strains of cells was discussed. These were as follows:

Strain D. This strain was never deliberately treated with any of the carcinogen. It was considered as the control strain of the series. Strains H, J, L, N and O were treated for 6, 32, 111, 184 and 406 days respectively with a concentration of 1 microgram of the carcinogen in each cc. of the culture medium. The last cultures were removed from carcinogen September 16, 1942, since which time all cultures have been carried on in its absence. At from 25 to 90 days after the

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initial addition of carcinogen to the culture medium, a characteristic morphological change in the pattern of the cultures was observed. The cells treated for 6 days at the glass interface of the culture became more laterally coherent and showed a shortening of their terminal processes. At stage J, 32 days in carcinogen, the cells were even shorter and showed a marked tendency to form lateral processes; at 111 days cell cohesion was even more exaggerated with a tendency to form rather epithelial-like sheets although these sheets continued to be very thin. With the cultures treated 184 days, that is strain N, the sheets became thicker and the cells showed even closer cohesion and in instances some traces of rounding. There was also a definite increase in cell granularity; in cultures which had been treated for 406 days, the cells were granular, growth was very much retarded, the cells were very closely coherent, often into massive sheets, and frequently showed tendencies to rounded or spherical shapes.

At about 120 days after the last cultures of these treated cells had shown clearly recognizable morphological alterations, the presumably untreated control cultures also began to show a very slight alteration of the same general type as was seen in the treated cells. The degree of change in these control cells has been even less than that seen in the cells treated for the shortest interval of time, that is 6 days, in the carcinogen. At the present time no definite cause can be assigned for this change in the presumably untreated control cultures, but the possibility of their having received an accidental trace contamination of the carcinogen used must be most seriously considered.

An examination of cells at the fluid interface of the cultures showed a very different but no less characteristic series of cell changes. For instance, strain L cultures characteristically showed numerous extremely large cells; strain N showed cells peculiarly discrete from each other laterally, while strain O showed peculiarly "stringy" cells, very unthrifty in appearance. The extreme lateral cohesion seen at the glass interface was lacking.

Considering only the cultures deliberately treated with 20-methylcholanthrene, it appears that this compound did act directly or indirectly in some way to induce a recognizable, characteristic, morphological alteration in the cells. These changes seemed to affect most or all of the cells in the culture. They increased in severity the longer the cells were subjected to the 20-methylcholanthrene.

These strains of cultures have to date been carried nearly 4 years

since their removal from 20-methylcholanthrene. At the present time strains J, N and O have shown no regression to their original normal morphology, but they have shown certain further changes in their respective morphologies which may be due to either cell selection or to further progressive change in tissue culture. Even so they still show many morphologic characteristics peculiar to their respective morphologies a short time after removal from the 20-methylcholanthrene. Strains D, H and L have shown no recognizable trend of morphologic change from the degree of alteration they showed a short time after their removal from carcinogen, that is at December 17, 1942, approximately 3½ years ago. Allowing, generously, 5 days for each intermitotic interval in the cells of these cultures the evidence is that these cell strains have shown an extraordinary stability in their altered morphology for well over 200 consecutive cell generations.

When cultures of each of these cell strains were injected into mice of C3H strain, tumors arose at the site of injections. From the work of Dr. Nettleship and Dr. Dunn it appears at the present time that the degree of cellular anaplasia of these tumors was directly correlated to the time the respective cell strains from which the tumor strains arose were subjected to 20-methylcholanthrene. Neither the rate of growth of the tumors nor the percentage of injections which gave rise to tumors was directly correlated with time of exposure to the compound however. Both of these showed a very high peak at stage D, dropped through H to a low at about stage J, rose to a secondary peak at stage L, and dropped to an extreme low at stages N and O. The shape of this curve was also reflected in the curve which showed the vascular reaction of the hosts of the tumors as determined by Dr. Glenn H. Algire, in the curve which showed the increase in the width of the original cultures in vitro, and in the curve which showed the orientation of the Golgi apparatus within the cell, as determined by Dr. Albert J. Dalton. From these various data it seems that not only the morphology of the cell was altered but that the carcinogen had induced a profound change in the basic physiology of the cells, and that this change was demonstrable in both in vivo and in vitro.

The tumor strains which arose from injection of these cell strains (D, H, J, L, N and O) were carried on by serial subinoculation of C3H mice. At about the 53rd generation in vivo, Miss Shelton in our laboratory, re-grew, in culture, cells from these respective tumor

strains. These cells were carried in vitro in these "recovery" cultures for about 6 months. In these "recovery cultures" tumor strains D, H, L, N and O reproduced in tissue culture, after more than 50 generations in vivo, characteristic designs of growth which were indistinguishable from the original characteristic designs of the respective cell strains from which these tumors arose. Strain J, however, on re-culture underwent a curious transformation. After a short preliminary interval in culture, a large percentage, or practically all the cells of the culture went over into extremely large or giant cells.

The analysis of these studies is as yet incomplete and as yet only very sketchy data from many of them can be given. It is expected that within a short time a complete publication on this work will be sent out through the Journal of the National Cancer Institute.

This research is merely one instance in which long-term tissue culture procedures have been used in the study of cellular growth and differentiation. For many studies simpler procedures are more satisfactory than long term tissue culture techniques. Often short-term tissue cultures of freshly explanted tissues carried in culture for only a limited time are entirely adequate or even more satisfactory. For some purposes, however, the study of such long term cultures under controlled culture conditions offers peculiar advantages in the examination of phenomena of cell differentiation and growth.

At the present time, however, there are several technical obstacles the removal of which would greatly increase the usefulness and accuracy of long term tissue cultures. For instance: (1) The ability to grow cultures from a single cell would give a control over the uniformity of cell strain which does not now exist, and would allow more accurate identification of cell strains, as for instance in that group of cells we now loosely designate as "fibroblasts". (2) The elaboration of a chemically defined culture medium would allow the continuous growth of cells for extended intervals under far more accurately controlled culture conditions. This would represent a tremendous technical advance since it would allow a far greater accuracy of research in cell nutrition; it would probably allow growth of cultures with much greater ease and reproducibility and would open up the way for many unexplored applications of tissue culture procedures. (3) More accurate and easier methods for routinely and accurately calculating rates of cell proliferation in culture would offer tremendous assistance in a study of the influence of culture conditions on the cells.

It is recognized that in general the technical details for the successful growth and study of substantial numbers of long term tissue cultures for extended periods of time requires considerable equipment, technical assistance and an extensive training both in operation of equipment and in establishing a fundamental working knowledge of the behavior of living cells in vitro. It appears to the author, however, that long term culture procedures have much to contribute to our knowledge of the cell and its behavior. What it has contributed in a substantial number of studies in the past is almost certainly merely an introduction to its possibilities which can be realized only when a larger body of workers have adequate facilities to carry on such long term cultures and become skilled in their use. In this respect the author would call attention to how few graduate students are now being trained to have a real theoretical grasp and a practical working knowledge of such long-term tissue culture procedures. The question is suggested for the consideration of the audience as to whether provision should not in some way be made in institutions offering graduate courses for the adequate training of a more substantial number of workers in this line.

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CONTRIBUTIONS TO THE STUDY OF AUXIN PHYSIOLOGY

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INTRODUCTION

It is now generally appreciated that a group of plant hormones, the auxins, play an important role in the regulation of plant growth and development. Early critical work in this field was done by Went (1928) who showed that in the case of the etiolated oat seedling, a growth regulating substance is formed by the tip of the seedling coleoptile. This substance is transported from the apical region to the lower regions of the coleoptile and the presence of this substance is essential to growth in length of the organ. Since growth of the coleoptile is essentially owing to elongation of preformed cells, early workers were naturally inclined to the view that the growth substance in question might represent a cell elongation hormone. Chemical identification of substances active in causing cell elongation in the oat coleoptile was accomplished by Kögl and coworkers (1933, 1934) who reported the isolation from urine and other sources of three substances, auxins a and b and the chemically non-related indole acetic acid, all active on the coleoptile. Although none of the substances was isolated from growing plant tissue, Kögl et al. felt on the basis of qualitative tests that the natural growth hormone of higher plants is probably auxin a or b (Kögl et al., 1934) and that indole acetic acid might represent primarily a metabolic by-product of microorganisms (Kögl and Kostermans, 1934). The effectiveness of the auxins in regulating cell elongation may be demonstrated in a variety of ways. One method which has been of particular utility in the problems to be discussed below is the excised coleoptile section technique (Bonner, 1933). Sections about 5 mm. long of actively growing oat coleoptiles are excised and suspended in solution. If the medium is water alone, the sections grow slowly and essentially cease elongation after a few hours. If, however, the solution contains an auxin, for example, indole acetic acid in a concentration of 1 mg. per liter, growth of the sec-

tions is rapid and indeed the sections may increase in length by 50 per cent. or more within 24 hours. The effectiveness of the auxin may be further increased by addition of a carbohydrate substrate as sucrose to the growth medium (Schneider, 1938). By the use of this technique the influence of other added substrates and inhibitors on the growth reaction may be studied.

Early Work on Auxin Action

The mechanism by which auxin elicits its growth effect has been of interest since the first work on plant growth regulation. Heyn (1931) early found that coleoptiles supplied with auxin exhibit an increase in cell wall plasticity as compared with coleoptiles deprived and hence depleted of auxin. This effect is independent of whether or not actual elongation takes place and is definitely preparatory to growth. We may picture the effect of auxin on cell elongation then as a decrease in the wall pressure of the cell which results in an increased suction pressure and an increased water uptake. Since the effect is primarily on wall plasticity rather than on elasticity the resulting water uptake results in irreversible increase in cell size or growth. Deposition of new cell wall material, although it parallels elongation at ordinary temperatures, is not an integral part of the elongation process since at low temperatures growth under the influence of auxin may occur without any corresponding increase in cell wall material (Bonner, 1934). The relation of auxin to cell wall plasticity has been thoroughly confirmed by various workers and there can be no question but that this relation is a basic one in relation to plant cell elongation. The actual structural changes in the cell wall have been examined and shown to consist in a loosening of the points of interaction of the individual cellulose micelles of which the wall consists (Bonner, 1935). Other evidence indicates that the influence of auxin on cell wall plasticity is not the primary effect of the growth substance but that auxin effects rather fundamental processes in the cellular metabolism of which one expression may, under suitable circumstances, be the cell wall effect. Thus auxin is without effect on the wall plasticity of nonliving cells (Stewart, 1938). The effect is mediated by the protoplasmic activity of the cell. Of even more importance is the fact realized with increasing clarity after 1934, that the application of auxin to a plant tissue may result in any of a wide variety of responses depending on the tissue concerned and the particular

circumstances. Thus auxin is now known to evoke root formation (Thimann and Koepfli, 1935), bud inhibition (Thimann and Skoog, 1933), inhibition of root growth (Kögl et al., 1934), parthenocarpy (Gustafson, 1936), delay of abscission (Laibach, 1933), cambial activity (Snow, 1935), callus formation (Laibach, 1935) and increased rate of protoplasmic streaming (Sweeny and Thimann, 1938). It would appear probable that auxin acts on some basic and general cellular process and that the result of this action may be expressed in a variety of ways depending on the nature and condition of the tissue involved.

Auxin in Relation to Respiration

The basic and general cellular process whose relation to auxin has been most investigated is respiration. It was early discovered that when respiration is suppressed, for example by the absence of oxygen, auxin is ineffective in promoting growth (Bonner, 1933). Similarly inhibition of respiration by cyanide or urethane results in a wholly parallel inhibition of growth under the influence of auxin (Bonner, 1936). Whether or not the application of auxin results in any change in overall rate of respiration is however more controversial. Bonner (1936), Kögl et al. (1936) and Du Buy and Olsen (1940) found application of auxin in physiological concentrations to be without effect either on rate of respiration or on the respiratory quotient of oat coleoptiles. Commoner and Thimann (1941) and more recently Berger, Smith and Avery (1946) have reported on the contrary that application of auxin may result in small increases in respiratory rate. Thimann finds for example that auxin application may result in increases of the order of ten per cent, in respiratory rate. It is clear that in any case major effects on respiratory rate do not result from auxin application, even though inhibition of respiration results in a decrease in auxin induced growth. These relations may be viewed in either of two ways. It might be for example that the respiratory processes, while not involved directly in auxin action, do nevertheless produce a metabolite or a condition essential for auxin action. Thimann, on the basis of the small increases in respiration which he has found, has suggested alternatively that respiration may be divided into two components, a bulk respiration without particular significance for growth and a growth respiration comprising only a small part of the total but different in kind from the bulk respiration

and in which auxin participates directly in some manner. To support this view Commoner and Thimann (1941) have brought forward data concerning the relation of the four-carbon dicarboxylic acids to growth and respiration. These acids, such as succinic, malic, and fumaric, while they are without effect either on growth or respiration in the absence of auxin, do give small increases in both growth and respiration in the presence of auxin. Thus the growth respiration, it is suggested, may be one in which the four-carbon acids play a role while they do not play such a role in the bulk respiration. Further important evidence concerning the relation of growth and respiration is the fact reported by Commoner and Thimann (1941) that the enzyme inhibitor monoiodo acetate at a concentration of 3×10^{-4} M almost completely suppresses growth of the oat coleoptile while it is without important influence on rate of respiration. The growth inhibition may be offset by the further addition of 4-carbon acids. Three principal difficulties with this view are apparent at the present time. In the first place the influence of auxin in increasing the rate of respiration under physiological conditions has not been confirmed by all investigators. It was obtained in the case of Berger et al. (1946) with unphysiologically high concentrations of indole acetic acid (10 mgs. per liter). Secondly, the effects of the four-carbon acids are particularly apparent over long periods of time (10 to 40 hours), periods much longer than are needed to demonstrate the effect of auxin itself and the possibility of secondary effects should not be neglected. In the third place, although the biochemical mechanisms involved in the respiration of the oat coleoptiole have not been investigated in detail, still information from other species suggests that the oxidation of pyruvic acid through an organic acid or Krebs cycle may be the dominant pathway of respiration (Bonner and Wildman, 1946). Alternative pathways for the oxidation of hexose in plant metabolism being unknown, it is difficult to envisage the nature of the bulk respiration of the oat coleoptile which is assumed by Thimann to not involve the participation of the four-carbon acids.

The relation of auxin to certain individual enzymes of respiration has been delved into by Berger and Avery (1943). Auxin is without effect on the activity of the dehydrogenases *in vitro*. Coleoptiles treated with high concentrations of auxin for prolonged periods do show increases in content of alcohol and malate dehydrogenase of 100 per cent. or more. These increases in enzyme content do not appear how-

ever to occur to any marked extent in short term experiments with physiological auxin concentrations and their significance is at present unclear.

It is evident from the effects of the respiratory inhibitors that there is a close relation between growth under the influence of auxin and respiration. The intimate nature of this relation has been further emphasized by the work of Thimann. The manner in which the relation is established has not been revealed by investigations up to the present time.

The Auxin Protein

A fresh approach to the study of auxin action is suggested by the fact that auxin appears to be present in many tissues in the form of an auxin protein. Thus Skoog and Thimann (1940) and Wildman and Gordon (1942) have shown that auxin is present in bound form in *Lemna*, and in leaves and this auxin may be released by tryptic hydrolysis or by treatment with mild alkali. It would be of evident interest to know the nature of the protein to which the auxin is bound and to know what if any enzymatic properties are exhibited by the protein in question. This problem has been studied in the case of the proteins of the spinach leaf. The spinach leaf contains a relatively large amount of auxin of which 95 per cent, or more is present in the bound form and only 5 per cent, or less in the free, readily ether extractable, state. Suitable methods, which are described elsewhere (Wildman and Bonner, 1946) have been developed for obtaining the leaf proteins in the native state. These proteins may be divided following Chibnall (1939) into the green chloroplastic proteins and the cytoplasmic proteins. The chloroplastic protein, which is obtained in the form of grana or similar chloroplast fragments may be separated from the cytoplasmic fraction by high speed centrifugation. The cytoplasmic fraction may in turn be sharply fractionated into two principal fractions, fraction I, insoluble in 0.35 saturated ammonium sulfate and fraction II which is soluble. Fraction I which constitutes approximately 75 per cent. of the cytoplasmic protein, may be purified by reprecipitation and is obtained in the form of an electrophoretically homogeneous, brownish red protein of molecular weight (by osmotic pressure measurements) of 185,000. The proteins of fraction II on the contrary contain at least five electrophoretically distinguishable components. Fraction I yields an auxin on treatment with prote-

olytic enzymes or with hot alkali. The auxin cannot however be removed by dialysis, treatment with acid in the cold, etc. Fraction II does not yield auxin by tryptic or alkaline hydrolysis and appears to be auxin free. Thus all of the protein bound auxin of the spinach leaf with the possible exception of small amounts contained in the chloroplast, would appear to be associated with fraction I.

Fraction I possesses the enzymatic properties of a phosphatase. It rapidly hydrolyzes ATP and creatine phosphate, and attacks less rapidly glycerophosphate, fructose 1-6 diphosphate and phytic acid. It cannot phosphorylate hexose in the presence of ATP and is hence not a hexokinase. Fraction II on the other hand contains a variety of enzymatic activities including four dehydrogenases, polyphenol oxidase, peroxidase, aconitase, catalase and the enzyme described below which converts tryptophane to an auxin.

From the standpoint of auxin physiology, the auxin of the leaf is contained primarily in one, electrophoretically homogeneous fraction. This portion is an active phosphatase. Whether or not the auxin protein is in fact the phosphatase is an important question. Intensive fractionation has not thus far separated the two properties. No direct association of auxin with phosphatase activity has as yet been possible and there is no evidence with the spinach protein that auxin content is directly linked to phosphatase activity. Addition of auxin *in vitro* is without effect on the phosphatase activity of protein I, and it has not yet been possible to remove the auxin without denaturation of the protein. It is interesting to note that a close parallel between phosphatase content and growth activity of tissues has been found by Yin (1945) and others. The phosphatases studied by Yin are entirely similar to the phosphatase of fraction I in pH optimum, substrate specificity, lack of activation by Mg, and in other properties.

Experiments with *Avena* Coleoptiles

An independent indication of a relation between auxin and transformations of phosphorous compounds within the plant is supplied by experiments with oat coleoptiles. It has been found that oat seedlings do indeed contain a phosphatase similar in pH optimum and specificity to that of the spinach leaf fraction I. Excised sections of such tissue were floated on water with and without the addition of indole acetic acid in the concentration of 1 mg. per liter. After three hours when clearly measurable increases in growth had occurred, the

sections were ground and whole cytoplasmic protein of oat coleoptiles prepared along the lines used with spinach leaves. In two experiments the protein of coleoptile sections previously supplied with auxin was found to possess higher prosphatase activity and to contain 3-6 times more bound auxin than the protein obtained from coleoptile sections which had been depleted of auxin by being floated on water.

A further fact which suggests a relation between auxin action and phosphate metabolism in the plant is the fact that marked alterations in the amounts of the phosphate derivatives found in the coleoptile result from auxin application. Sections of coleoptiles were placed in auxin solution or in water and after three hours of treatment analyzed for the concentrations of various phosphorylated compounds according to the methods of Umbreit et al. (1945). These methods are primarily designed for animal tissue and microbial cells and since the possibility must be recognized that they may not apply accurately to the present tissue, the results are of necessity preliminary. It was found however that the auxin treated plants contained markedly more inorganic phosphate and markedly less fructose diphosphate and phytic acid as compared to the control tissue soaked in water. No marked change in labile phosphate was discovered. It should be noted that Albaum and Umbreit in their study of the normal growth of the oat seedling (1943) call attention to the rapid utilization of phytic acid during growth of the seedling. (The method used by Albaum and Umbreit in the hands of the present authors determines the total of phytic acid and fructose diphosphate.)

Yet a further indication of a close relation between phosphate metabolism and auxin action lies in the nature of differential growth inhibition caused by fluoride. This ion which is well known as an inhibitor of enolase and of phosphatases is also an efficient inhibitor of the growth of oat coleoptile sections under the influence of auxin since sodium fluoride in the concentration of 0.01 mg. per cc. brings about a 50 per cent. reduction in growth. This inhibition is not related to any influence on respiration since fluoride in this concentration has no detectable effect on rate of respiration. It is an interesting parallel however that the activity of the oat coleoptile phosphatase is also inhibited approximately 50 per cent. by 2×10^{-4} M sodium fluoride. The parallel between fluoride sensitivity of growth and phosphatase activity holds true over a wide range of inhibitor concentrations (Table 1).

TABLE 1
EFFECT OF VARYING CONCENTRATIONS OF NaF ON GROWTH, RESPIRATION AND PHOSPHATASE ACTIVITY OF OAT COLEOPTILE. TWO HOUR EXPERIMENTAL PERIOD.

Concentration of NaF	% inhibition of coleoptile growth	% inhibition of respiration	% inhibition of phosphatase
0.01 mg./cc	50	0	51
0.1 "	94	34	74
1.0 "	100	70	90

Three kinds of experiments with oat coleoptiles yield then evidence indicative of a relationship between phosphatase, metabolism of phosphorylated compounds and growth under the influence of auxin. These lines of evidence, e.g. increased bound auxin and phosphatase activity in the proteins of auxin treated coleoptiles, increased utilization of fructose diphosphate and phytic acid in auxin treated coleoptiles, and inhibition of growth by fluoride concentrations which inhibit phosphatase but are without effect on respiration, are at best only suggestive. They suggest that we may utilize as a working hypothesis the concept that auxin plays its role as a component of an enzyme involved in the metabolism of the phosphorylated compounds.

A WORKING HYPOTHESIS OF AUXIN ACTION

The facts presented above concerning the auxin protein and concerning phosphate metabolism in the plant do not as yet compel the choice of any one picture of how auxin acts. It may be nevertheless of value to suggest such an overall picture which is in accord with these facts and which will be of use as a working hypothesis in guiding the design of further experiments. Auxin appears in the plant in a variety of forms, including a form in which auxin is bound to a protein to form, it is assumed, an auxin protein.. This auxin protein is an enzyme and in the specific case here studied appears to be a phosphatase. Even though this phosphatase as studied *in vitro* appears merely as an agency for the breakdown of organic phosphates, it is entirely likely that in the cell, in an organized system, breakdown of high energy phosphates may be coupled with other systems. The energy contained in the phosphate bonds attacked by the auxin enzyme may be transferred to the synthetic reactions which constitute growth, perhaps synthesis of new cytoplasm, of new nuclear material, of other growth substances, etc. It is certainly to be expected that the receptor of this energy, the synthetic reaction to which the auxin

enzyme reaction is linked, may be quite different in different tissues, a supposition which would aid us in interpretation of the fact that auxin application may result in different responses in different tissues. This working hypothesis would also suggest the nature of the relation between respiration and auxin action since if the auxin protein acts primarily on the high energy phosphates as ATP, these must in turn be supplied by respiration. This hypothesis here intersects that of Thimann and Commoner since it is known from the work of many investigators that respiration and the oxidation of the plant acids in particular is attended by the formation of high energy phosphate bonds. The present hypothesis suggests then that the auxin protein is a portion of the system whereby energy produced in respiration is shunted over and made available to the synthetic processes on which growth depends. The exact nature of the shunt as well as the identification of the individual synthetic processes involved are however yet to be investigated.

Biogenesis of Auxin

The question of how auxin is formed in the plant is closely related to the problem of how auxin functions, since both problems involve a knowledge of the identity of the native plant auxin. An additional community of interest will become apparent below. Early studies of the precursor of native auxin revealed that auxin activity is produced oxidatively from the amino acids of peptone (Bonner, 1932) by cultures of *Rhizopus*. Thimann has shown (1935) that *Rhizopus* converts tryptophane, presumably by oxidative deamination, decarboxylation, and oxidation, to indole acetic acid. The same holds true for a wide variety of microorganisms. Skoog (1937) who has studied auxin biogenesis in the oat coleoptile, has found that the coleoptile does contain a material, itself inactive, as a growth regulator, but which can be converted to an active auxin by the auxin generating tissue of the coleoptile tip. Skoog also produced evidence compatible with the view that oat coleoptiles may be able *in vivo* to convert tryptophane to an active auxin, presumably indole acetic acid (see also Went and Thimann, 1937). Since Skoog considered with Kögl that the native auxin of higher plants is auxin a or b he did not propose that tryptophane might be a native auxin precursor. Evidence presented in detail elsewhere (Wildman, Ferri and Bonner, 1946) shows that the spinach leaf possesses an enzymatic system for the

conversion of tryptophane to an active auxin. This system may readily be obtained and studied *in vitro* as well as *in vivo*. Since the auxin produced has the chemical and physiological attributes of indole acetic acid it may be assumed in a preliminary way that the auxin produced is indeed this compound although the product has not been isolated and identified. On the basis that the product is indole acetic acid the yield from tryptophane is low, of the order of 0.01 to 0.1 per cent. of the tryptophane supplied. It will be of great interest to discover whether this enzyme system is widespread in nature and whether the production of indole acetic acid from tryptophane is of widespread occurrence. With regard to the spinach leaf itself however, since the leaf does contain the auxin producing enzyme and since tryptophane as well as tryptophane containing proteins are unquestionably included among the cytoplasmic constituents, it would seem most probable that the production of indole acetic acid from tryptophane may normally occur. The free auxin of the spinach leaf as well as the auxin obtained by hydrolysis of the auxin protein both appear by qualitative physiological and chemical tests to be closely similar to indole acetic acid.

Berger and Avery (1944) have studied in detail the characteristics of a material contained in corn seeds which liberates indole acetic acid on treatment with alkali. The name precursor has been applied to this material and obviously it may function as a precursor of auxin in the seed in the sense that the compound, which in some ways resembles a protein, may contain bound indole acetic acid. The indole acetic acid contained in the Berger and Avery complex must have itself arisen however by some other means, possibly by that outlined above. The auxin proteins of the leaf have similarly been referred to as auxin precursors and here again it is true that auxin may be liberated by hydrolysis of the auxin protein. If the principal function of the auxin protein is to act as an essential enzyme it is unlikely that it plays an important role as a precursor of free auxin. In any case the indole acetic acid of the plant whether free or combined must have its ultimate origin in some such reaction as that involving tryptophane. It would seem most appropriate to reserve auxin precursor for compounds from which auxin may be synthesized, produced *de novo*, and to apply other terms to compounds in which indole acetic acid is merely bound or stored.

Inactivation of Auxin

It has long been known that the activity of auxin in solution or contained in agar blocks may be reduced by contact with tissue from certain species or with plant extracts. Van Overbeek (1935) in particular has sought to link increased auxin destruction with restricted growth in the case of dwarf strains of corn. It is possible to show that tissues of various species including etiolated seedlings of bean, pea and sunflower contain highly active enzymes which inactivate indole acetic acid (Larsen, 1940; Tang and Bonner, 1947). The enzyme from pea seedlings in particular has been concentrated and studied. Action of this enzyme is inhibited by cyanide and CO, the latter inhibition being reversible in the light. The enzyme needs no dialyzable cofactor. It attacks the indole acetic acid molecule via the sidechain, with the uptake of oxygen and evolution of CO_2 and yields a product inactive as an auxin but containing the indole nucleus intact. The enzyme is highly specific and attacks only indole acetic acid among substrates investigated. Thus indole butyric and naphthalene acetic acids which are active as growth substances although they do not occur in nature are not attacked by the enzyme. An interesting feature of this enzymatic system is the widespread existence in plant tissues of a heat stable alcohol soluble inhibitor of the enzymic reaction. The exact role of this enzyme in the auxin economy of the plant is still to be worked out although it is evident in a general way that it may function in regulating the concentration in the tissues of native or applied auxin. An obvious application of the enzyme is however its use as an aid in the diagnosis of the chemical nature of native plant auxins.

The Nature of the Native Plant Auxin

The problem of the chemical nature of the native plant auxin was in general considered a closed and finished chapter ten years ago. Auxins a and b, closely related and both highly active, were isolated and brilliantly characterized by Kögl and his group. These compounds were obtained not only from urine but from malt and corn oil. Indole acetic acid on the other hand was obtained from some urines as well as from microorganisms. Differentiation of auxin a, b and indole acetic acid in crude extracts was based on (1) the diffusion constants of the active principle. Auxin from *Avena* coleoptiles for example was

shown by Went (1928) to have the diffusion constant expected for auxin a or b rather than that expected for indole acetic acid. (2) The differential destruction of the active principle in acid or alkali. Auxin a is readily destroyed in hot alkali (Kögl et al., 1934), indole acetic acid is readily destroyed in hot acid and is more stable in alkaline solution while auxin b is destroyed in both conditions. By this criterion also the native auxin of the *Avena* coleoptile would appear to be auxin a. Application of the two methods was early made to the auxin of a variety of tissues and results concordant with those expected for auxin a were obtained for the growth hormone of oat, *Vicia,* corn, and other seedlings (Went and Thimann, 1937). On the other hand auxin tentatively characterized as indole acetic acid by these same qualitative tests has been reported in recent years from tomato (Locke et al., 1939), spinach (Wildman et al., 1946), *Elodea* (Van Overbeek, 1940), radish (Stewart, 1939) and other higher plants. The identification of unknown auxins by acid-alkali destruction tests or by determinations of diffusion constants has obvious unassessable liabilities due to possible interference by other materials such as the naturally occurring auxin inhibitors shown to be present in corn meal, in *Vicia* seedlings (Goodwin, 1939) and in leaves of radish and other crucifers (Stewart) as well as to possible formation of auxin from related compounds (Larsen, 1944). An example of the complications which may arise became apparent during the course of work with the auxin of the spinach leaf. Although pure indole acetic acid is inactivated by prolonged heating in dilute alkali, it is not vigorously attacked by the same treatment in the presence of spinach leaf protein. The auxin is apparently protected from destruction by the protein or its breakdown products.

In an analysis of the phototropic relations of seedlings, Van Overbeek (1936) made a significant contribution to the question of the identity of native plant auxin. He showed that crystalline auxin a (Kögl) and indole acetic acid differ in their response to light *in vivo*. In oat plants supplied with auxin a, growth is reduced by light while if the plant is supplied with indole acetic acid such reduction does not occur. Van Overbeek further showed that the auxin of corn meal behaves like auxin a rather than like indole acetic acid. The auxin of corn seeds has now been isolated by Haagen Smit et al. (1941), Berger and Avery (1944) and by Dandliker (1945). In every case the auxin obtained was indole acetic acid. It would seem then that

the impure indole acetic acid of corn seeds behaves in Van Overbeek's test just as did Kögl's crystalline auxin a. It is doubtful then that Van Overbeek's test can be used, as it once was, to support the thesis that native plant auxin is not indole acetic acid. The isolation of auxins a or b from plant material has not as yet been announced by investigators other than Kögl et al. Bergren (1941) in an attempt to repeat the isolation of auxins a and b from malt and corn oil by the Dutch workers (1934) was unable to find samples of these materials with more than approximately one twelfth the activity reported by Kögl. The samples used by the Dutch workers were exceptional in containing unusually high concentrations of active material. This active material present in high concentration may or may not have been identical with the usual native hormone.

The fact that enzyme systems both for the formation and for the inactivation of indole acetic acid have been found further indicates that this compound is of metabolic importance in the higher plants. This coupled with the fact that indole acetic acid has actually been isolated from higher plant materials shows that in any case the view that indole acetic acid is not a normal plant product (Kögl et al., 1934) is no longer tenable. Whether or not indole acetic acid is not only present in the plant but is also actually a native plant hormone is a further and as yet unsettled question. The specificity of the indole acetic acid inactivating enzyme has been alluded to above. It has not been possible to test the action of the enzyme on an authentic sample of pure auxin a or b so that it cannot be known with certainty whether or not the enzyme attacks this material. It is of interest to note however that the enzyme rapidly attacks the auxin of the oat seedling. This fact coupled with the observation of Skoog that the oat coleoptile is capable of producing an active auxin from tryptophane leaves ground then for the suggestion that even the auxin of the oat coleoptile may in fact also turn out to consist in part at least of indole acetic acid.[1]

It must be recognized that a great weight of evidence concerning differences in behavior between indole acetic acid and the native auxin of particular species of higher plants has been adduced by a variety of workers. This short review has shown however that in other cases

[1]Recent experiments have shown the presence of both the enzyme for production of indole acetic acid from tryptophane and the enzyme for inactivation of indole acetic acid in the oat coleoptile.

indole acetic acid is itself a native constituent of higher plants and the possibility must be recognized that this may well be widely true and that the substance may also be a native hormone.

Resumé

This review has concerned recent work on the biogenesis, inactivation, and function of auxin in the plant. The views expressed are of necessity tentative and open to revision in particular since they represent views derived from work still unfinished and actively under way, and they are presented in the hope that they may prove a stimulus to further investigation. A schematic representation of the several systems which have been studied is given in Figure 1. Tryptophane may be converted to an auxin, presumably indole acetic acid, in the spinach leaf and possibly in the oat coleoptile. The auxin so produced may then undergo any one of a variety of reactions. It may in the first instance be inactivated enzymatically. Or, it may be combined with other materials to form inactive storage forms represented perhaps by the corn endosperm material of Berger and Avery (1944). Yet another possibility is represented by combination to form an auxin protein such as that possibly represented by the protein of fraction I of spinach leaves which would appear to be an active enzyme and which may represent the form in which auxin plays its role in the regulation of plant growth. Tryptophane which is envisaged as the ultimate precursor of indole acetic acid has been shown by Tatum and Bonner (1943) to arise in *Neurospora* by the union of indole and serine. It will be of interest to discover whether a similar relation exists in higher plants. Tryptophane functions not only as a precursor of indole acetic acid but also as a component amino acid of the plant proteins, for example of protein I. The tryptophane thus incorporated into the proteins is however in a state of dynamic equilibrium with the free tryptophane since as shown by the work of Vickery et al. (1940) the plant proteins are in a state of constant flux, and for the same reason indole acetic acid combined in protein may well be in dynamic equilibrium with the free auxin of the plant.

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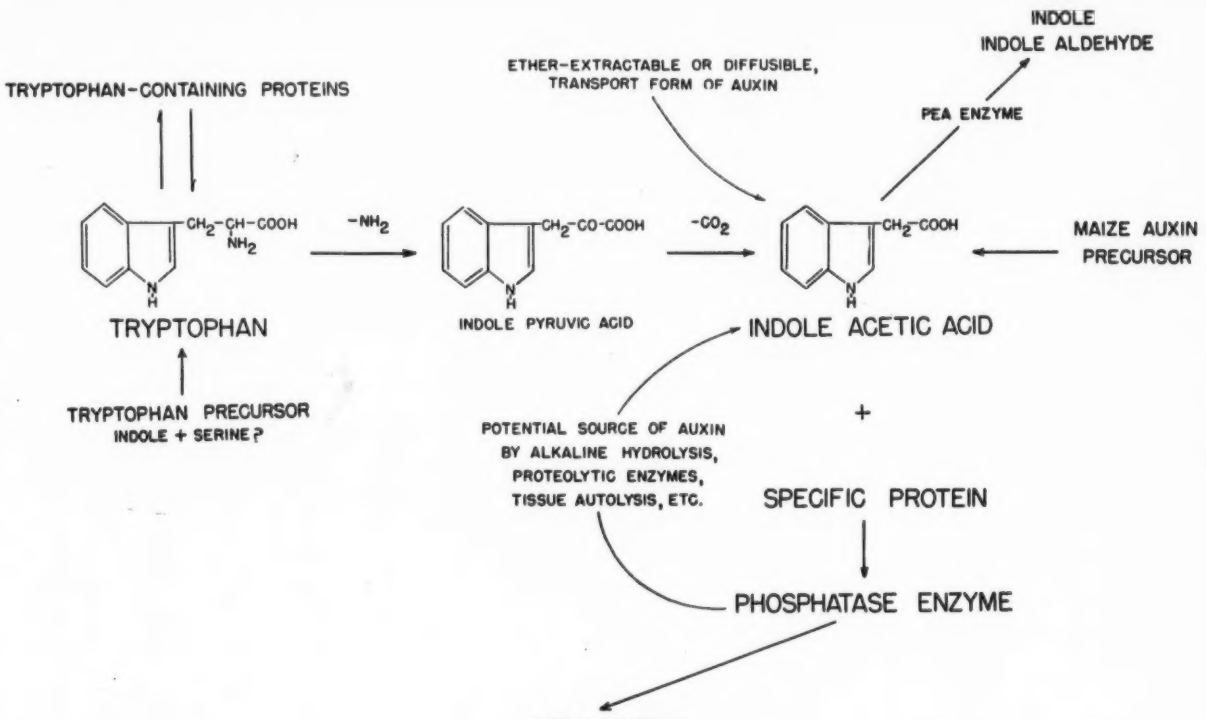

FIGURE 1

A Tentative Summary of Possible Interrelations of the Various Forms of Auxin Together with Suggested Mechanisms of the Formation and Destruction of the Material

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AGRICULTURAL PROBLEMS IN GROWTH AND AGING[1]

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The student in agriculture investigates such aspects of growth and aging as promise eventually to yield bigger, better, and more profitable crops of plants, animals, and such of their products as meat, milk, eggs, wool, and muscular work (in horses and mules).

The special difficulties in agricultural investigation consist in the many uncontrollable factors—ecological, reproductive, hereditary, nutritional, engineering, social-economic—and in the financial and methodologic obstacles consequent to the sheer size of the experimental individuals or populations.

SPECIFIC GROWTH PROBLEMS

The agriculturist defines "growth" not only as "developmental biosynthesis" but also as the profitable production of beef steaks and pork chops, milk and eggs, beans and potatoes, peaches and tomatoes, wool and cotton, flowers, race horses, Easter rabbits, Christmas trees, and so on. The following are some examples of growth problems in agriculture.

The preferred market weight of pigs is 225 pounds. Unimproved pigs may attain this weight in 12 months at an approximate cost of 8 pounds feed per pound gain in body weight; but some attain it in 6 months at a cost of 4 pounds feed per pound gain, demonstrating that two pounds pork may be produced in half the time where one was produced before.

The preferred market weight of beef steers is 900 pounds. Unimproved animals attain this weight in about 2 years at a cost of perhaps 8 pounds TDN per pound gain in live weight, but some attain it in one year at a cost of 4 pounds TDN per pound gain.

[1]Before the decision was made by the Society to publish complete proceedings of the Symposium, Dr. Brody had made arrangements to have his paper published elsewhere. It appeared in *Science*, Vol. 104, pp. 307-321, without illustrations. The present abbreviated form with illustrations has been prepared as an abstract for these proceedings.

An unimproved cow may produce 1,000 pounds of milk a year, but some produce 42,000 pounds (perhaps 55 quarts a day). Such cows demonstrate the plasticity, productive range, and potentialities in this productive category in that over 50 quarts of milk may be produced where 1 quart was produced before.

An unimproved fowl may lay 30 eggs a year beginning at 1 year of age, but some begin laying at 5 months of age and produce 360 eggs the first year. Such birds demonstrate the possibility of producing a dozen eggs where one was produced before.

The increase in corn yield by the use of "hybrid" seed varies with climatic and soil conditions, averaging about 25 per cent; this relative increase is slight but the absolute increase on a 3 billion dollar crop is huge. Greater yield, earlier maturity, and resistance to disease (wilt, mildew, smut, smudge, blotch, etc.) were obtained in many plants by the use of "hybrid vigor".

The phenomenon of real hybrid vigor is best known in the mule, superior to either mare or jackass for hard labor in hot climates. Resistance to heat is, however, also shown in crosses between some European breeds of cattle evolved in cool climates and Asiatic or African cattle evolved in hot climates.

Individual differences in rates of fattening are common in many species, including men and mice. Physicians attributed them in man to differences in food consumption in relation to muscular activity. What causes such differences in food consumption? "Yellow gene" mice show an unusual tendency to rapid fattening. How does the "yellow gene" accelerate fattening? It is also known that administration of thiouracil (goitrogenic substance) or stilbestrol (estrogenic substance) and certain injuries to the brain increase the fattening rate.

A major growth problem in biology is to discover the mechanisms of these individual variations and a major problem in agriculture is to discover animals and plants that grow rapidly and efficiently to the preferred size and quality and that transmit consistently these characteristics to their progeny. Unfortunately, only a few of the rapidly growing animals produce rapidly growing progeny, and these few can be detected by progeny tests alone. But by the time adequate progeny tests are completed, the original stock is usually no longer available or has become infertile or impotent.

To utilize fully the few superior progeny-tested animals, agriculturists developed methods for artificial insemination which enable the

utilization of one sire for perhaps 100 times as many females as by the natural method.

Attempts are also being made to "rejuvenate" old animals, at least temporarily, by medication and by diet. For intance, F. A. E. Crew reported (1924) that he "rejuvenated aged fowls (5 to 8 years) through thyroid medication." Recently C. W. Turner, of the Missouri Station, confirmed Crew's results by applying such medication on a large scale to fowls. Five-year-old birds fed thyroactive protein produced approximately 30 per cent more eggs than control birds. Berliner and Warbritton (1937) and Bogart and Mayer (1946), of the Missouri Station, also reported favorable results of thyroxine administration on the fertility of old rams and on fertility during the period of "summer sterility". M. A. Goldziehr reported (1946) a rejuvenating effect of topical applications of estrogens on senile genital mucous membranes and even on skin. C. W. Turner is administering estrogens to fowls in the attempt to prevent the deterioration of their oviducts at the end of the laying season.

No successful method has been developed for *early* recognition of future productive ability in farm animals comparable to the methods that have been developed for *early* recognition of intellectually gifted children; yet this is urgently needed. The various categories of productivity—growth, fattening, milk production, egg production, wool production, endurance (in horses and mules)—may well be correlated with the levels of limiting catalysts (hormones, enzymes, etc.), nutrients, cardiorespiratory behavior, metabolic rates, electrical configuration (of Burr), and so on. These levels may perhaps in turn be used to predict future performance in the young.

Aging Problems

Next to growth rate, the aging rate is the most important factor in the overall economy of animal production. Dairy cattle do not pay for themselves in milk and calves until they are about four years old, and the longer thereafter they maintain a satisfactory yield in milk and calves the greater the clear profit on the growth investment. The same holds true for poultry, for workstock (horses and mules), and for all breeding stock, especially for those that are progeny tested.

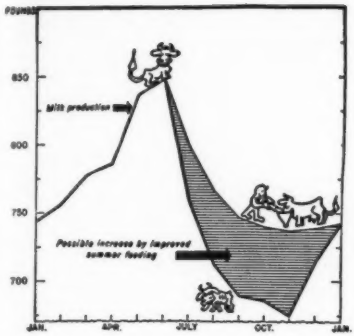
About 1940 R. Schoenheimer, employing isotopes of hydrogen and nitrogen as tracers, observed that there is continuous renewal of body nitrogen. But as this metabolic interchange occurs at an ever decreas-

ing rate with increasing age, the *proportion* of young tissue declines with increasing age. This decrease in young tissue or increase in old tissue presumably constitutes aging. Would thyroxine, for example, which apparently accelerates the metabolic exchange, also delay the aging rate—that is provided it was administered at certain optimal levels—or would it, on the contrary, accelerate aging because of the extra metabolic strain it imposes? What is the bearing of "Rubner's energetic law" of aging on the use of thyroxine? Similar questions might be asked about other hormones, for example, sex hormones, and also about other processes, such as lactation, ovulation, and neoplastic growth: what, for example, would be the effects of thyroxine and thiouracil administration on cancer growth? Would it accelerate or retard it? It has already been noted that thyroprotein appears to "rejuvenate" old fowls and old rams. But what are its long range effects on longevity, on neoplastic growth, on the heart, and what are the mechanisms of these effects?

Climatic Factors in Relation to Growth

The internal physico-chemical environment of the body—its several metabolic rates, electro-magnetic configurations, concentrations of catalysts, nutrients, etc.—is dependent not alone on heredity and on nutrient supply but also on the external environment, including temperature, humidity, air movement, radiations, barometric pressure, and ecologic factors, some of which are illustrated by the following examples.

Rats that secrete 9.9 mcg. thyroxine at 1° C. secrete but 1.5 mcg. at 35° C. (Atwood and Dempsey, 1943); fowls that secrete 12 mcg. thyroxine in winter secrete but 8 mcg. in summer (C. W. Turner, 1946); rams that are fertile in cool weather are sterile in hot weather (Berliner & Warbritton, 1937, and Bogart and Mayer, 1946). Increasing the environmental temperature from 70° to 90° F. reduces the calcium level in fowl's blood by 30 per cent (Conrad, 1939) and also the size of the egg and the thickness of its shell (D. C. Warren, 1940). Increasing environmental temperature from 40° to 95° F. reduced the daily milk yield in dairy cows from 29 to 17 pounds (Fig. 1), the casein from 2 to 1.8 per cent, the solid non-fat from 8.1 to 7.6 per cent, and increased the rectal temperature from 101° to almost 104° F. (Regan and Richardson, 1938). The growth rate of two-weeks-old chicks is greatest at 70° F. and declines rapidly with



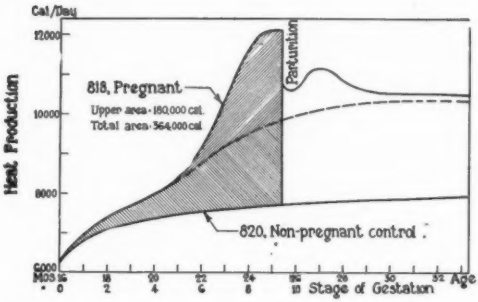


FIGURE 1
MILK YIELD IN RELATION TO ENVIRONMENTAL TEMPERATURE AND FEED SUPPLY
(Courtesy of the U. S. Department of Agriculture.)

FIGURE 2
HEAT INCREMENT OF GROWTH AND GESTATION

both rising and declining temperatures (Kleiber, 1934). The same is apparently true of growth in all farm animals. There is an optimal temperature for growth and for all productive processes. One of the major problems in agriculture is to map the influence of temperature and other climatic factors on growth and other productive processes. The influence of climatic factors on aging of homeotherms is not known although there is a large literature on small poikilotherms.

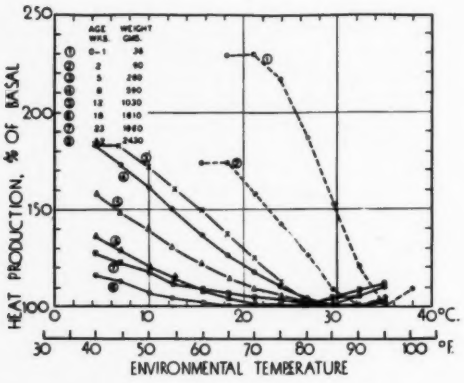
It is generally known that disinclination to muscular exertion declines rapidly with rising humidity and temperature above 70° F. This explains the "poise" of the Southern ladies and gentlemen, the "laziness" of the Southern white and colored "trash", and the low productivity, including growth, of all farm animals under conditions of high temperature and humidity.

The reason for the decline in growth and other activities with increasing temperature above about 70° F. is that all activities—including maintenance, feed consumption, waste excretion, reproduction and growth (Fig. 2), milk production, egg production, wool production, and especially muscular work—occur at an energy cost; they involve the production of huge amounts of heat, which must be promptly dissipated else the body temperature will rise. This heat dissipation becomes ever more difficult, especially in non-sweating species, as the environmental temperature approaches that of the body. If the

animal cannot give off the heat rapidly enough to maintain its body temperature normal, it reduces the level of its metabolic processes by depressing its endocrine activities; it reduces, for example, thyroxine secretion as previously explained; and it reduces food intake and all productive processes. This result could be predicted from the principle of homeostasis. The long-range effect of high temperature is to reduce the body size and to slenderize it since the smaller and the leaner the body the greater the surface area per unit weight, and heat is dissipated by way of the surface.

The manner of reaction to environmental temperature varies with many factors, including age, body size, body build, efficiency of the sweating and cardiorespiratory mechanisms, activity of the nervous and endocrine systems, amount of insulation (fur, feathers, fat), protective coloration, and so on.

The influence of age, body size, and environmental temperature on heat production is illustrated in Figs. 3 to 5. The smaller and the



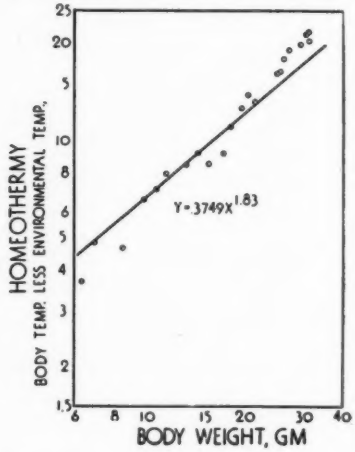


FIGURE 3

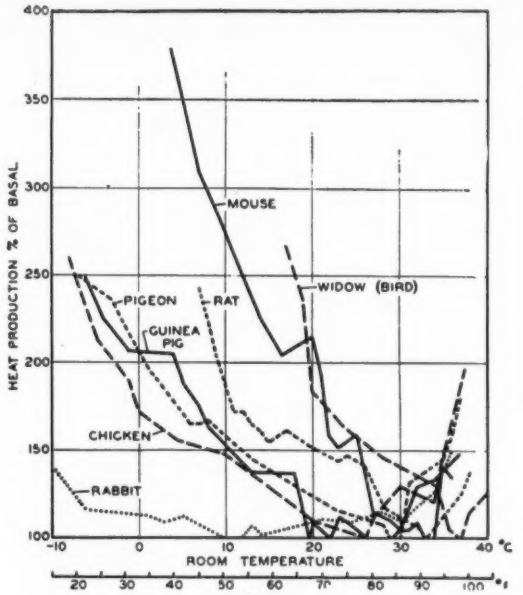
HEAT PRODUCTION IN CHICKENS OF DIFFERENT AGE AND WEIGHT AT DIFFERENT ENVIRONMENTAL TEMPERATURES

This figure was prepared on the basis of data and charts by H. G. Barrott and E. M. Pringle, *J. Nut.*, **31**, 35, 1946. Note that the lowest heat production (thermal zero or thermoneutrality) is at higher temperature in small-young than in large-older birds; and that the larger birds are less sensitive to cold weather than the smaller birds.

FIGURE 4

DEVELOPMENT OF HOMEOTHERMY (ABILITY TO MAINTAIN CONSTANT BODY TEMPERATURE) IN A GROWING RAT

The data were obtained and the chart prepared in 1942 by Eugene B. Brody in the writer's laboratory.



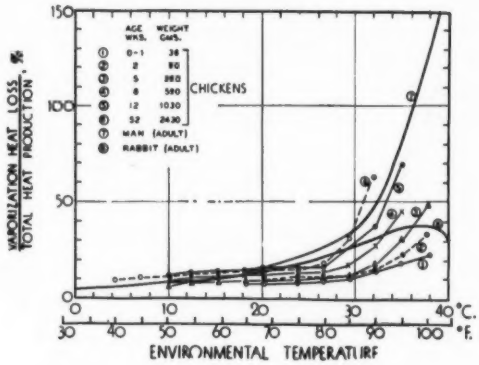


FIGURE 5

THE RELATIVE INFLUENCE OF ENVIRONMENTAL TEMPERATURE ON MATURE ANIMALS OF DIFFERENT SPECIES, DIFFERING IN BODY SIZE

Note that the large rabbit is less sensitive to cold than the small mouse—just as the small chick (curve 1) is less sensitive than the large fowl (curve 8) in Fig. 3. Curves based on data by E. F. Terroine.

FIGURE 6

While man can dissipate by vaporization several times the heat production (curve 7), chickens, rabbits, etc., can dissipate by vaporization but half of the heat production; hence the much greater hot-summer distress of non-sweating species (swine, sheep, cattle) than of sweating (man, mule, ass). Curves on chickens prepared on the basis of Barrott's and Pringle's data l.c. in legend for Fig. 3.

younger the animal the more sensitive it is to environmental temperature, especially to cold.

The influence of sweating ability on ease of keeping the body temperature normal is illustrated in Figs. 6 and 7. Non-sweating animals (which include virtually all farm species) can dissipate by vaporization only a little over half of the heat produced by the body. Sweating species such as man, mule, and ass (curve 7, Fig. 6) can dissipate by vaporization several times as much heat as is produced by the body. Non-sweating species attempt to compensate by panting (Fig. 7).

The agricultural importance of the temperature problem is due to the facts that: our best farm livestock evolved in cool climates—for example, dairy cattle in Holland, Denmark, Scotland, and the Channel Islands; their sweating mechanism—the chief cooling device in hot weather—is therefore undeveloped (Figs. 6 and 7); for hygienic or mass-production reasons they are prevented from using natural cooling methods, such as seasonal migration, use of mud wallows by swine and cool streams by cattle; they are concentrated in the Middlewest and other regions subject to very high summer temperatures. Selection of farm animals for high productive potentialities is not helpful if prevented from realization by unfavorable temperatures.

As previously mentioned, one possible method of increasing resistance to high temperatures is biological, by breeding the non-sweating cattle evolved in cool regions with the sweating breeds evolved in hot regions; the other is engineering, involving the development of cooling devices. Fig. 8 is a diagram of a "climatic laboratory" for dairy cattle now under construction on the University of Missouri experimental farm with full control of temperature, humidity, air movement, and light.

Growth and Aging Standards

In order to investigate quantitatively the influence of the many factors on growth and aging rates, it is desirable to have typical or average values, so-called "standards", and their development and interpretation is one of the investigational tasks.

There are generally accepted yardsticks for overall growth, especially for growth in weight, and for aging of special functions, such as of milk and egg production. We have not, however, yet learned to measure the growth and aging rates of work ability in horses and mules.

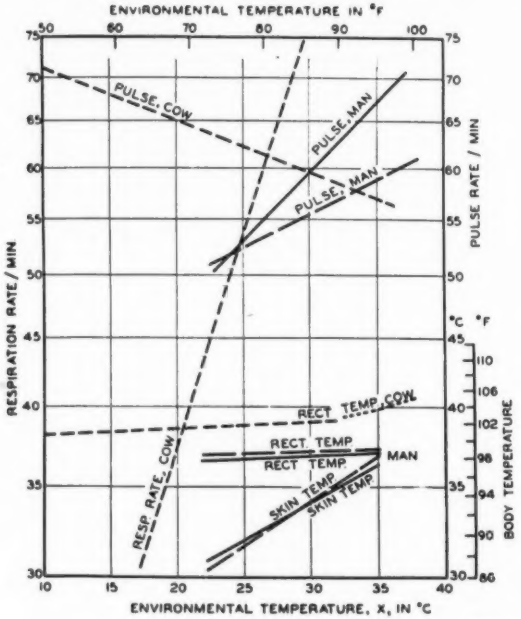


FIGURE 7

Note how the respiration rate rises with rising temperature in (non-sweating) cattle. Whereas it remains constant in (sweating) man. Chart based on data by J. D. Hardy and E. F. Du Bois (man), and W. M. Regan and G. A. Richardson (cow).

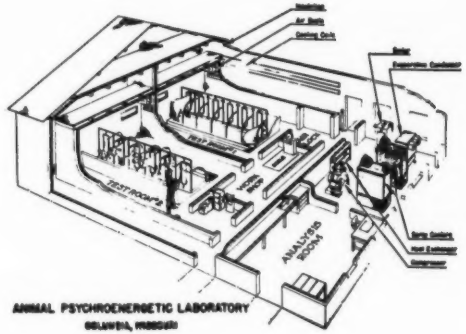


FIGURE 8

Diagram of climatic laboratory for dairy cattle being built cooperatively by the U. S. Department of Agriculture (Agricultural Engineering Division) and the Missouri Agricultural Experiment Station. Courtesy of U. S. Dept. of Agriculture (BPISAE).

The available standards themselves, however, seem to change with time and circumstance. For instance, present-day white rats are larger and approach mature size at a different rate than those of 25 years ago because of selection and difference in method of feeding and housing. The same holds true for farm livestock and, indeed, for children.

Standards are also needed for relative (allometric or heterogonic) growth, the relation of part to part and part to whole. For instance, the value of a pig is dependent on the *relative* amounts of its bacon, ham, loin, etc.; the value of a cow on the *relation* of milk yield to body size (maintenance cost), and so on.

Understanding is needed of the significance of the allometric relationships. For instance, J. Hammond and C. P. McMeekin (1928-40) demonstrated differences in economy of total and relative growth by manipulating the speeds of growth at different ages in certain ways; they demonstrated that a basic difference between a "modern" and a "primitive" pig is in the bodily proportions at different ages.

The relation between monetary profit and biologic efficiency also needs investigation; for profit depends not only on biologic efficiency of the process, but also on many economic factors. For instance, two 800-pound cows producing as much milk and at the same energetic or biologic efficiency as one 1600-pound cow are not as profitable because the labor cost of milking, feeding, cleaning, housing, etc., is nearly twice as great for two 800-pound cows as for one 1600-pound cow.

In connection with the need for growth standards one thinks of the needs for sharply defined *biologic units* of mass and time. For instance, the metabolic rate per unit weight of a 0.05 pound mouse is about 20 times as great as that of a 1200-pound cow; so is the rate of approach to mature body size about 20 times as great in the mouse as in the cow. This means that the *physiological* significance of a *physical* unit of weight or time in a mouse is about 20 times that in a cow. Physical units of time and weight are therefore ambiguous when applied to biological processes in different species. There is need for clarification of *physiologic* units of mass and time, for defining clearly the meaning of "mouse time" and "cow time" (E. W. Sinnott) and "mouse weight" and "cow weight".

These, in brief, are a few of the growth and aging problems in agriculture which are perhaps not without interest to "pure biologists", physicians, and oncologists.

VIRUS TUMORS IN PLANTS

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There are several viruses which cause overgrowths in plants. Ordinary tobacco mosaic virus, for example, is usually not regarded as a virus producing overgrowth, because it does not cause such reactions on most species susceptible to it; but it regularly produces leafy outgrowths, called enations, from the under sides of the leaves of *Nicotiana paniculata* L. and *N. tomentosa* Ruiz. and Pav. Furthermore, one variety of the virus has the unusual property of producing enations on infected tomato plants. Sugar beet curly-top virus is distinguished by the production of little papilla-like protuberances on the under sides of the veins of leaves of susceptible beet plants.

However, of the plant viruses that produce overgrowths, only two are known to the writer that produce definite galls or tumors. These are the viruses of Fiji disease of sugar-cane and wound-tumor disease. Fiji disease occurs in certain islands of the Southwest Pacific and in Australia (Lyon, 1921) and is known only on sugar-cane. Wound-tumor disease occurs in the United States (Black, 1944) and is capable of affecting many different kinds of plants. Wallaby ear disease of corn in Australia (Schindler, 1942) and swollen shoot of cocoa in the Gold Coast of Africa (Anonymous, 1937) may also be diseases of this type.

The wound-tumor disease. Wound-tumor disease is caused by the virus *Aureogenus magnivena* Black. As a rule, wound-tumor is a systemic or generalized disease, but sometimes it remains localized in a part of the plant for months. Infected plants usually have irregularly enlarged leaf veins, and tumors on the roots. In some species, like sorrel (*Rumex acetosa* L.) diseased plants may produce papillae or even small tumors on the enlarged veins. The tumors commonly occur on the roots but in sweet clover (*Melilotus alba* Desr.) they also occur on the stems. The root tumors in different species vary in size from mere pustules to globose growths about 1 cm. in diameter and in number from one or a few to so many that the root is almost completely covered with them (Fig. 1). Old root tumors rot, so that

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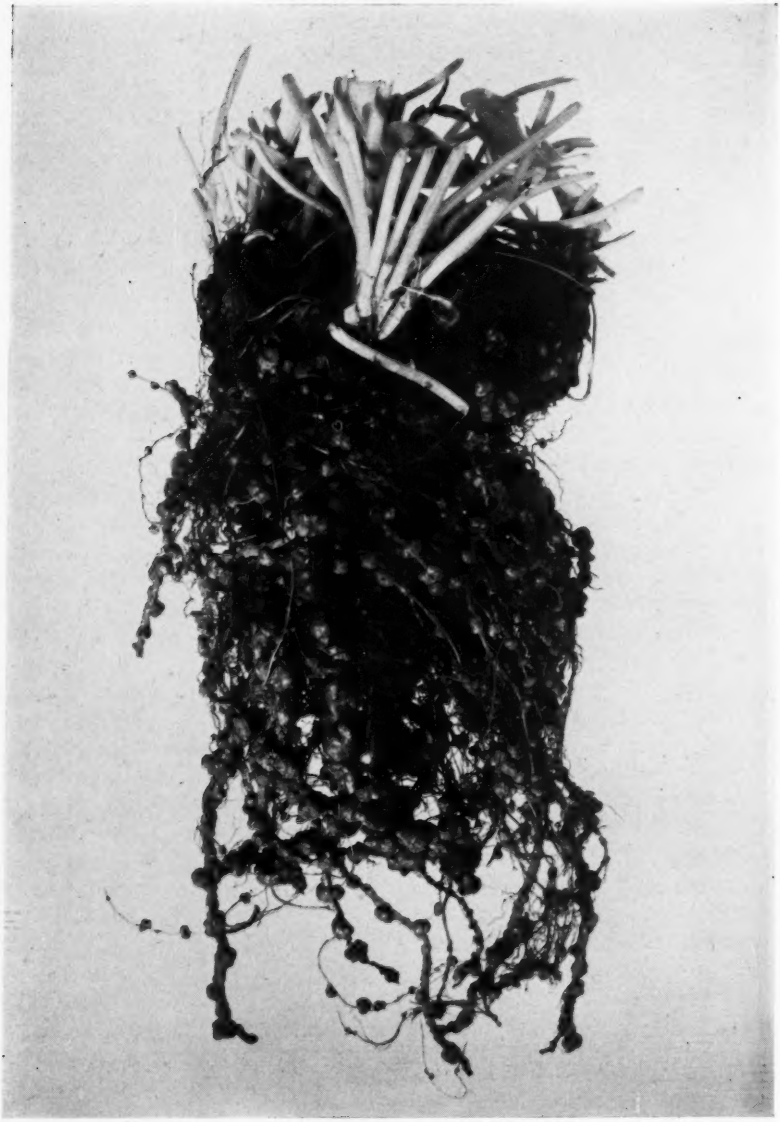

FIGURE 1
AN OLD ROOT OF SORREL BADLY AFFECTED BY WOUND-TUMOR DISEASE (x 0.9)
(Photograph by J. A. Carlile)

there may be very little root system left in advanced stages of the disease. The plant may die or, due to the remarkable regenerative ability of plant parts, it may manage to survive for a long time in an unthrifty condition.

Examination of the tumor tissue under the microscope shows that the cells differentiate but that they are disorganized and sometimes arranged in whorls. There are many centers of growth, each of which may be delineated by meristematic cells and surrounded by a layer of crushed cells. No organisms have been observed in the diseased tissue. Non-granular, non-vacuolated, smooth spherical bodies staining intensely with safranin, may occur in the cytoplasm of the tumor cells.

Induction of tumors by wounds. Although the virus usually invades the whole plant the tumors are local in character. Observations indicate that wounds play an important part in starting the tumors in the diseased plants. Tumors have been observed on stems of sweet clover (*Melilotus alba* Desr.) exactly at the point where the stems rested on the rims of the pots in which the plants were growing (Fig. 2). In sweet clover, tumors are common in the axils of branches,

FIGURE 2
THE LOCATION OF THE STEM TUMOR ON THE LOWER RIGHT HAND BRANCH OF THE SWEET CLOVER PLANT SUGGESTS THAT IT AROSE AS A RESULT OF A WOUND TO THE BRANCH BY THE RIM OF THE POT (x 0.65)
(Photograph by J. A. Carlile)

natural points of stress. In sorrel (*Rumex acetosa* L.) the old leaves absciss at the crown of the plant and the crowns of diseased plants are often tumorous. Tumors have been observed growing from severed veins in wounded sorrel leaves. The correlation between root tumors and lateral roots is striking, the tumors usually being in immediate proximity to a lateral root so that the root tumor pattern and the lateral root pattern are practically identical. In the higher plants susceptible to this virus the lateral roots arise in the pericycle and wound the endodermis, cortex and epidermis in growing out through the mother axis. The evidence therefore indicates that these wounds initiate the tumors in infected plants. In contrast with lateral roots, the lateral appendages of the stem originate from embryonic centers near the surface and do not cause wounds as roots do. This may account for the relative scarcity of stem tumors.

The observations on naturally occurring wounds have been confirmed by experiment (Black, 1946). Insect pins 0.25 mm. in diameter were used to make single punctures in the internodes of healthy and diseased sweet clover stems. From 387 such punctures on virus-infected stems 175 tumors developed, whereas from 505 corresponding wounds on virus-free plants no tumors developed. In old stem tissues of infected sweet clover, pin punctures rarely induce tumors; in young tissues each pin puncture may yield a single tumor; while each puncture in a node near the growing point may give several tumors.

Tumor tissue cultures. Sorrel root tumor tissue has been grown in White's medium (White, 1943), slightly modified, for two years. It grows slowly on this medium, the volume of tissue doubling in the best cultures about every 3 weeks. Better growth could probably be obtained with a medium especially adapted to sorrel tissue. The tissue cultures are free of cultivable organisms as indicated by the absence of any growth of microorganisms on White's medium itself or in dextrose broth solutions to which ground tissue from culture is added. However, when such tissues are successfully grafted back to sorrel roots the stock plants develop the systemic tumor disease, showing that the virus is still present in such tissue cultures.

The tissues in culture have never become organized into roots, stems, or leaves, and this suggests that the tumor tissue is capable of indefinite growth without the development of such organs. This interpretation is supported by the results of grafting sweet clover

tumors from plant to plant in series. Cells descended from those in the first scion continued to develop as tumor tissue in subsequent transfers and did not produce roots, stems or leaves. These results suggest that the limited growth of the tumor on the mother plant is probably due to the inability of the disorganized transport system of the tumor to transfer food sufficient for growth more than a short distance. This explanation is supported by the fact that tumors are larger than usual on the single stem of a sweet clover plant pruned so that all the food from the top passes the tumors and by the fact that tumors sliced off a stem renew their growth from the remnants left in the stalk.

Differences in host susceptibility. When sweet clover plants grown from commercial seed were inoculated with the wound-tumor virus, the resulting infections varied widely in the size and number of the tumors. Such variation might be due to variation in the host or in the virus or in both. To test the influence of variation in the host, six sweet clover plants differing considerably in their morphological characters were selected. Cuttings were made from each to establish six groups of plants, the plants within each group being propagated vegetatively and thus being genetically identical. When the plants were inoculated it was found that the tumor reaction within each clone was quite uniform but that the reaction in different clones varied from a mild infection with very minute tumors that might easily escape detection to very severe infection with roots a mass of tumors. This result indicates that heredity of the host plays an important part in the tumor reaction.

Transmission of the virus. In a limited number of experiments wound-tumor virus could not be mechanically transmitted from plant to plant when juice from a diseased plant was rubbed on leaves of healthy plants. Diseased and healthy plants may grow in close proximity without the infection spreading. The causal virus is transmitted by certain specific agallian leafhoppers, *Agalliopsis novella* (Say) and *Agallia constricta* Van Duzee; another agallian leafhopper, *Acerata-gallia sanguinolenta* (Provancher) will not transmit it. The virus has many of the attributes of other viruses transmitted by leafhoppers. The insect vectors cannot infect other plants immediately after feeding on diseased plants. The virus must incubate in the insects for several days before the insects become infective. It may be that the virus multiplies in the insect during this time as some other leafhopper-

borne viruses are known to do. The insect is not detectably injured by the presence of the virus. The virus does not pass from parent insects to offspring through egg or sperm and this indicates that the virus in nature must alternate between plant and specific insect vector in order to survive.

Host range. In a preliminary survey, using the insect vectors, the virus was shown capable of infecting 47 species in 20 families of plants (Black, 1945). Probably many more species are susceptible in other genera and families.

Conclusions. In any studies on wound-tumor disease as related to its general significance for tumor research the following facts seem to be of particular importance:

No causal micro-organisms can be detected in tumor tissue by microscopic examination or culture methods.

The disease is not contagious but requires the intervention of specific insect vectors for its spread.

Apparently several factors play a vital secondary role in starting tumor growth in plants invaded by the virus. Wounding is one of these factors; heredity of the host plant is another. The age of the wounded tissue also appears to be important.

The disease is caused by a virus that may invade the whole plant or may be localized in a part of it for months.

The tumor tissue never reverts to normal and, in spite of the remarkable regenerative capacity of plants, the disease does kill some plants and holds others in an unthrifty state until they die from other causes.

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RECENT EXPERIMENTAL CONTRIBUTIONS TO BRAIN MORPHOGENESIS IN AMPHIBIANS[1]

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Without any doubt, the formation of the vertebrate brain is the kernel of the morphological problems offered to our investigation in higher Chordates. It is a considerable task to understand the relations between the embryo and the brain emerging from it, and we can only begin to perceive the general program of that new field of research.

The first question it offers to the investigator refers to the existence of an enlarged rostral portion of the neural plate, a feature so closely connected with primordial morphogenesis. Secondarily, at the stage when the neural tube and the neural crests have been formed, we have to consider the modelling of the primary vesicles according to the well-known pattern, viz., the relative individuation of the secondary brain vesicles, the appearance of the olfactory lobes, of the optic vesicles, of the infundibulum, and, later on, of the pineal gland, together with the budding of the macro- and microplacodes. The student of these processes must bear in mind that they are combined with a progressive and considerable growth of the neural tissue, and also that they lead to the acquisition of a symmetrical organization. He should finally attempt to explain the complementary modelling of the associated organs, their cytological differentiation, the conformation of the cephalic sense organs, the cranial nerves, the cerebellar and cerebral cortices, the internal nuclei and the various fiber tracts connecting them. All of that continuous and intricate construction is necessary to provide the head of the embryo with the morphological substrate of its future nervous activity. In those

[1]I wish to express my appreciation to Dr. J. Auer, head of the Anatomy Department of the University of Ottawa, and to Dr. E. K. Hall, of the Anatomy Department of the University of Louisville, for their kind assistance in the revision of this paper.

Editor's note: In the original plan of publication it had been intended to accompany Professor Dalcq's paper with short statements of the recent work of Drs. Nieuwkoop and Trampusch to which he referred. The statements submitted, however, were so extensive that it would be inappropriate to include them in the published proceedings of this Symposium. It is hoped that they will appear elsewhere in the near future.

processes, primary morphogenesis, individuation, growth and differentiation are intimately linked, and also correlated with events occurring in neighboring tissues.

It is beyond the scope of this paper to present a complete account of the data relating to the head and brain problem. Excellent reviews have summarized this, in particular those of Mangold on "Determination" ('28), and the "Eye" ('31), Harrisons' classical paper on the neural crest ('38), Adelmann's remarkable one on cyclopia ('36), J. Ariens Kappers' ('41) equally valuable one on cephalic placodes. But any student of these reviews will realize that they only give explanations for partial problems, and leave the general one, with its numerous aspects, almost intact. This is why it seems valuable to consider here certain experimental results which perhaps give a new hint for a better understanding of brain structures. But it should be emphasized that we are still far from covering the whole field offered by an analytic program such as we have tried to sketch in the foregoing lines. In particular, the problem of neural differentiation in the strict sense will have to be neglected, and the intense growth which is so characteristic for the brain wall and some of the sense organs will only be alluded to.

In the *terminology* used here, the three primordial layers are to be called entoblast, chordo-mesoblast and ectoblast. The ectoblast is subdivided into neurectoblast and epiblast by the process of neurogenous induction or evocation. After having contributed to the formation of the placodes and possibly some dermal constituents, the epiblast is finally reduced to the epidermis. This nomenclature has the advantage of following the sequence of the successive segregations step by step; ectomesoblast is, of course, equivalent to ectomesoderm.

I. First Steps in the Experimental Analysis

Except for a few attempts to relate the brain with suprablastoporal territories in the amphibian embryo (A. Brachet, '23; L. Desclin, '27), the causal analysis of brain structures has its foundation in Spemann's memorable discovery of the primary, neurogenous induction ('24). The last piece of research that great pioneer was able to perform on the organizer ('31) introduced, but not without caution, the concepts of head and trunk organizers, and thus laid the basis for the investigations now in progress. Nearly equally important for further

advances were the first experiments on the ear (literature in Yntema, '33, and in Dalcq, '33; Trampusch, '41; Zwilling, '41) and the eye (literature in Adelmann, '36; Alderman, '38). But, for the time being, the relations between the various results remained unclear. In spite of its interest, the distinction between head and trunk organizers did not lead to the experimental dissociations which should have been obtained, had it been exactly founded. The head organizer did not, or only exceptionally, induce a brain to the exclusion of spinal cord, and, conversely, the trunk organizer proved able to induce a brain, at least when grafted in the head region of the host (Spemann, '31). Of course, the necessity of the head organizer being present to obtain a normal primary head was demonstrated by E. K. Hall ('37), working in Spemann's laboratory. The substitution of an advanced dorsal lip for a young one resulted in a considerable reduction of the brain. But, again, the reciprocal experiment did not produce cerebral structures in the trunk region. There was something unsatisfactory indeed in the proposed distinction.

This situation was considerably improved when, on the basis of his LiCl intoxications and microsurgical operations, F. E. Lehmann ('38) recognized that the true distinction was to be made between the prechordal and the chordal parts of the archenteric roof. He therefore proposed dividing the brain into an archencephalon and a deuterencephalon. As will appear from the following, my observations have led me to recognize the same divisions. But the terminology chosen by Lehmann is not advisable, since "archencephalon" has previously been used by v. Kuppfer ('06) for the entire primary brain vesicle, and also because the hind part of the brain does not have, as far as I can see, a secondary character. Because of the importance of a correct nomenclature, I have suggested ('46) the terms of *acrencephalon* and *chordencephalon*. The first term recalls the participation of that region of the brain in the formation of the "acromerite", a concept of A. Brachet ('14), which indicates a remarkable anticipation of the present views. The second emphasizes the relation with the cephalic part of the notochord, but must be understood as implicating also the parachordal mesoblast, which is indeed equally important.

II. Recent Experimental Advances

Such was, in its main features, the situation when during this very year (1946) new results have emerged, which in my opinion throw some light on the brain problem. Some of them were obtained, quite independently, by Dutch workers, a second part is my own contribution, while a third one, which will be mentioned only briefly, concerns unpublished data of studies now in progress in my laboratory.

1. *Trampusch's* data. In the laboratory of Professor Woerdeman (Amsterdam), Trampusch has studied the effects of carefully dosed X-irradiation on the Axolotl embryo at the pregastrula and at the neurula stage. The most remarkable fact concerning the brain is that adequate irradiation at the younger stage results in a microcephaly in the etymological sense of the word, i.e., a considerable reduction in size of the optic vesicle, together with a shortening of the brain itself, but without cyclopia or monorhinia. After irradiation at the neurula stage, the microphthalmia is no longer obtained, but the shortening of the cerebral parts is still present.

2. *Nieuwkoop's data.* In the zoological laboratory of Professor Raven (Utrecht), Nieuwkoop has repeated the fundamental experiment of Spemann, i.e., grafting a young dorsal blastopore lip into the ventral marginal region of another gastrula. The only difference is that being orginally interested in the germ cells, the author has raised his larvae to an advanced stage. He was struck by the fact that they can be classified into a continuous series on the basis of the degree of completeness of the brain in the secondary embryo. The striking point in his results is that the brain appears to be reduced in its fore part only, in each case at a definite level. This does not mean that the anterior part present, e.g., the mesencephalon or the diencephalon, is not incompletely formed. But all parts posterior to it, of the brain stem, as well as of the spinal cord, are perfectly normal. There is neither an anomaly of the notochord nor of the myotomes, as would be found if the microcephaly had been caused by LiCl intoxication. It is important to note that in cases of the greatest reduction, the anterior limit of the formed part of the rhombencephalon may be situated at the level of the auditory vesicles, or even as far caudally as the IXth and Xth nerves.

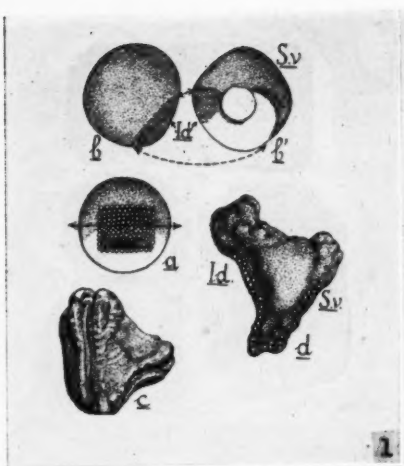
3. *Personal contribution.* My own results in this field will be given in greater detail. They have been obtained by the use of a rather peculiar operation called *latitudinal translocation.*

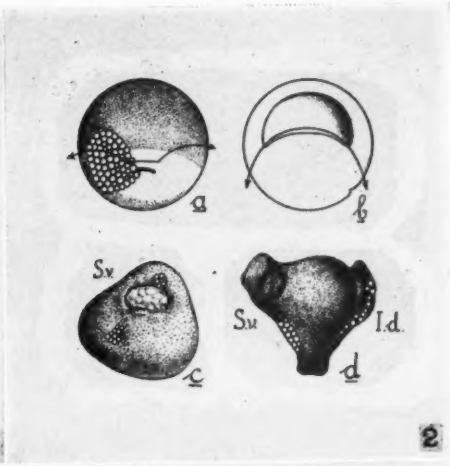
A. *The method.* An advanced blastula or a young gastrula of the anuran *Discoglossus pictus* is given a large patch of Nile blue stain on its dorsal side. Then the embryo is cut completely through from the dorsal to the ventral side, following some approximate level of the anlagen plan. This isolates the region of the animal pole, together with a variable, more or less controllable amount of marginal material. This animal part of the embryo is now rotated 180° and exactly superimposed on the vegetal part. Healing occurs rapidly, a small amount of injured material being cast off along the line of coaptation. The two now opposed parts of the stained region make it easy to follow the course of gastrulation. Since the dorso-marginal region has in most cases been divided by the incision, a double embryo is formed. The system of organs appearing on the original dorsal side will be called infero-dorsal (I.d.), the other one supero-ventral (S.v.). Since fixation is performed in such a way as to preserve the vital stain, the sections provide precise information as to the origin of the main organs (Fig. 1.).

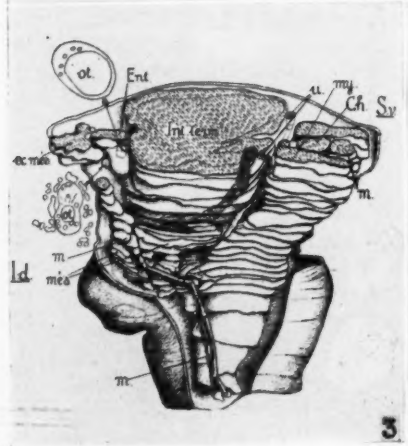
It must be admitted that this is almost a reconnoitering experiment. The level of the incision with regard to the various anlagen of the primordial organs can only be ascertained with relative certainty. The importance of the injured material is also difficult to estimate, though it may be stated that owing to certain favorable properties of the *Discoglossus* egg, it is much less than would be anticipated, and therefore practically negligible in many cases. In spite of these disadvantages, the method is reliable if it is completed by (a) an integral anatomical study of the entire double embryo, with all necessary graphic reconstructions; (b) careful consideration of the movements accomplished by the stained material, and of its final utilization; (c) a careful comparison of the results obtained by sectioning the embryo at different levels, asymmetrical operations being especially enlightening; (d) cautious interpretation in order to remain in accordance with the ascertained data of correlated experiments.

I have been conscious of these conditions as much as possible in the course of a long investigation begun in 1931, often interrupted but never abandoned, and dealing with more than a hundred double embryos. It now seems permissible to rely upon the results of that work for a new consideration of the brain problem.

Notwithstanding their difficulties, latitudinal translocations are especially fitted for this particular aim. They permit one to divide the







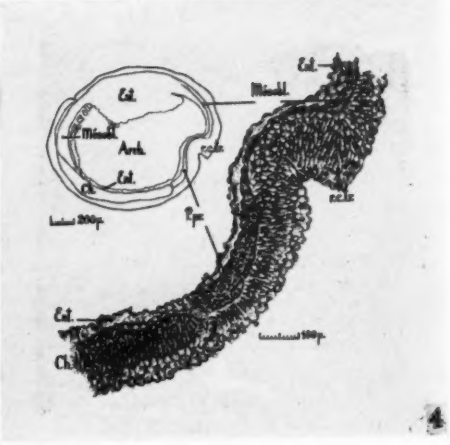


FIGURE 1
FIGURE 3

FIGURE 2
FIGURE 4

tiny anlagen operative in the induction of the brain, and to realize this result in various ways, according not only to the level of the incision, but also to its orientation in the depths of the marginal zone. Since nothing has been added to the morphogenetic system of the entire embryo, and since very little material has been injured or lost, we are really dealing with a change in significant topographical relations. The consequences may be examined from a kinematic as well as from a dynamic point of view. Such a study leads to a sure retrospective reconstitution of all the events subsequent to the operation.

B. *Results concerning the brain problem.* If the incision isolates the upper part of the dorso-marginal zone, i.e., presumptive chordo-mesoblastic material, or lateral parts of the same zone, which are presumptive mesoblastic material, such a system, whether supero-ventral or infero-dorsal, acquires only a truncal character, with a more or less typical spinal cord. The other system is complete, not only with respect to the brain but also the spinal cord.

When the incision is made somewhat nearer to the "center" of the dorso-marginal zone, whether more marginal, chordo-mesoblastic material is included in the S.v. system, or, conversely, more medial, mesoblastic material remains in the I.d. system, we again observe the formation of a trunk, but one which is prolonged forwards in the form of a cephalic swelling. This contains a more or less typical rhombencephalon, usually with a pair of auditory vesicles, sometimes with only one, or with an apical otocyst (Figs. 2, 3). The other system, whether it be I.d. or S.v., generally contains a complete embryo.

In these cases, and also in those to be considered later, a deficiency is eventually found in the auditory placodes or vesicles. They may present a balanced relation with the opposite system, the extreme difference being that one system possesses a right otocyst, the other a left one. Such a complementary situation has been interpreted (Dalcq, '33) as pointing to the existence of a special group of cells located in the parachordal mesoblast, and responsible for the induction of each auditory placode. It may be called, for convenience, an *oto-evocator*. Postponing the discussion of that special point (vide infra), it is important to state that in certain types of translocations one or more otocysts are eventually obtained in conditions which exclude the intervention of the primary oto-evocators.

The two preceding categories of operations already imply that the system containing the "center" of the dorso-marginal zone induces

a normal brain together with a spinal cord. A third category will group those cases in which this central region has apparently been divided between the two systems.

Any student of causal embryology will foresee that this center must be located in the presumptive prechordal material. It is well known that this material is found in the gastrula between the notochordal field above, the mesoblastic strips laterally, and the suprablastoporal entoblast below. Whether this tiny but active prechordal anlage is a part of the surface structure of the embryo, or, as seems more likely, whether it is located more deeply, under a thin layer of chordo-entoblastic cells, may be left open for discussion here. But it may be mentioned here that this small cluster of cells migrates with the upper part of the upper blastoporal entoblast to take a position in the most anterior part of the archenteric roof. There it separates by delamination from the entoblastic layer. It may be observed in the neurula stage as a flat plate of epithelial cells, somewhat thicker medially and posteriorly, and connected posteriorly with the notochord and also with the parachordal mesoblast (for it is broader than the notochord itself), and laterally with the head mesoblast (Fig. 4, 5). Naturally, the prechordal part of the brain neural plate is overlying the prechordal plate.

A more important point is to appreciate the degree of individuality and, if possible, the functioning of this small group of cells. It has been observed in several instances (literature in Töndury, '37; Dalcq, '40), that the simple excision of the dorsal blastoporal lip does not necessarily hinder the formation of a rather normal brain. The same is true for the *Discoglossus* egg (Dalcq, '40). It must be admitted that in these operations the prechordal material has been largely excised, but that it has been completed, or perhaps replaced, by neighboring elements. At any rate, there is certainly a definite limit to this regulative process, for a considerable number of similar operations yield embryos with defective anterior brains (loc. cit.).

The situation seems to be very similar to that found in translocation experiments. On the one hand, several cases show that no fore-brain manifestation is obtained if the "central" region of the dorso-marginal zone has not been included in the I.d. or S.v. system. On the other hand, when this center has probably been divided, fore-brain structures are present in both systems. Whether or not they are normal is a point to be considered later. But, in any case, it is not conceivable

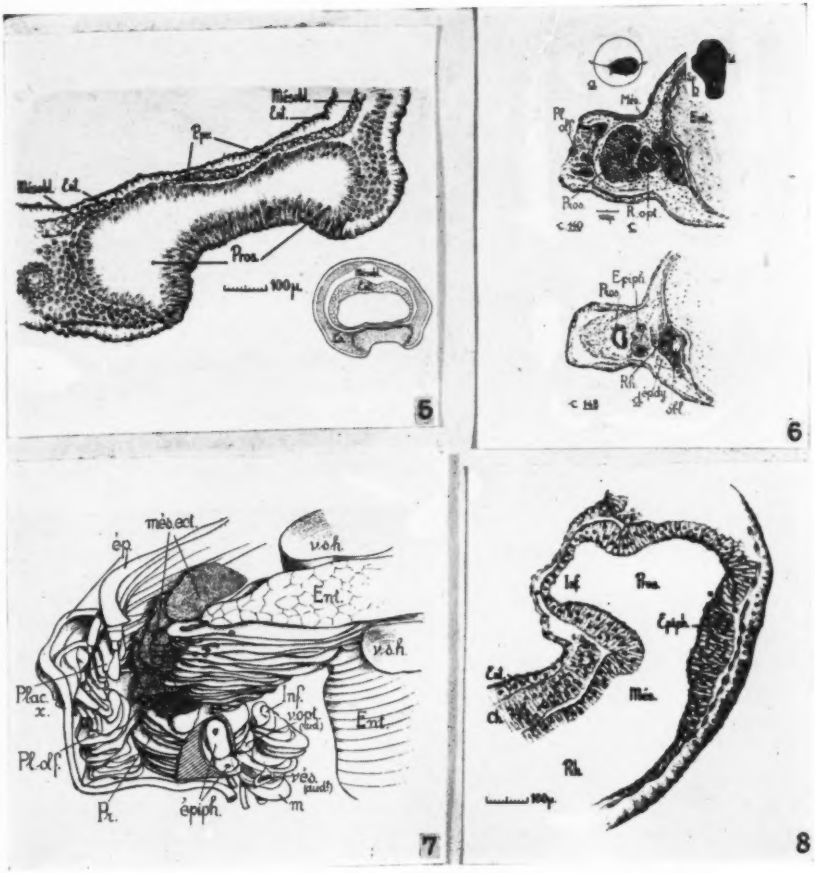


FIGURE 5
FIGURE 7

FIGURE 6
FIGURE 8

that such a delicate anlage could be divided by the instrument into two equal parts without some injury or dislocation. When we observe, as we exceptionally do, that two nearly normal forebrains have been obtained (Fig. 14), we are bound to admit that some regulation has taken place by the appropriate intervention of adjacent material. However, the situation created by translocations is certainly not so favorable for regulation as a simple local excision. The patch of prechordal cells is divided by a more or less latitudinal plane; ventral material—of ectoblastic or mesoblastic nature, according to the manner of sectioning the embryo,—is immediately superimposed on its dorsal part; conversely, the S.v. part is placed in contact with ectoblastic or mesoblastic (lateral plate) material. In fact, most cases are easily understood without any assumption of regulation. They offer to us a clear picture of the effects exercised by isolated parts of the prechordal anlage.

As for the morphogenetic activity of the prechordal plate, our previous information mostly concerns the neurula stage. The results of Mangold ('33), combined with those of the workers on the eye and nose problem, clearly indicate that the prechordal plate of the late gastrula is the evocator of the forebrain. But, as far as I am aware, no direct information is available for younger stages. Hence, some significance may be attached to the following fact: An operation performed on a gastrula with beginning invagination has resulted in a secondary structure with a distinct predominance of forebrain organs (olfactory, optic, pineal, prosencephalic structures), with a very feeble indication of the hindbrain parts (Fig. 6, 7). The interpretation is that the prechordal anlage was already located in the roof of the blastoporal pocket, and that a sufficient part of this material was translocated to the ventral side to act as a nearly specific forebrain evocator.

Since we have now considered the general kinematic and dynamic properties of the prechordal material, we may now proceed to the consideration of the forebrain structures obtained in double embryos, and of their reciprocal relations. Hence, we shall frequently mention an organ to which not much attention has been paid until recently by the embryologist, i.e., the pineal gland or epiphysis.

It is of course well known that this organ is formed rather late as a bud proceeding from the roof of the prosencephalon. v. Kuppfer ('06) long ago called attention to its constancy in Vertebrates. In

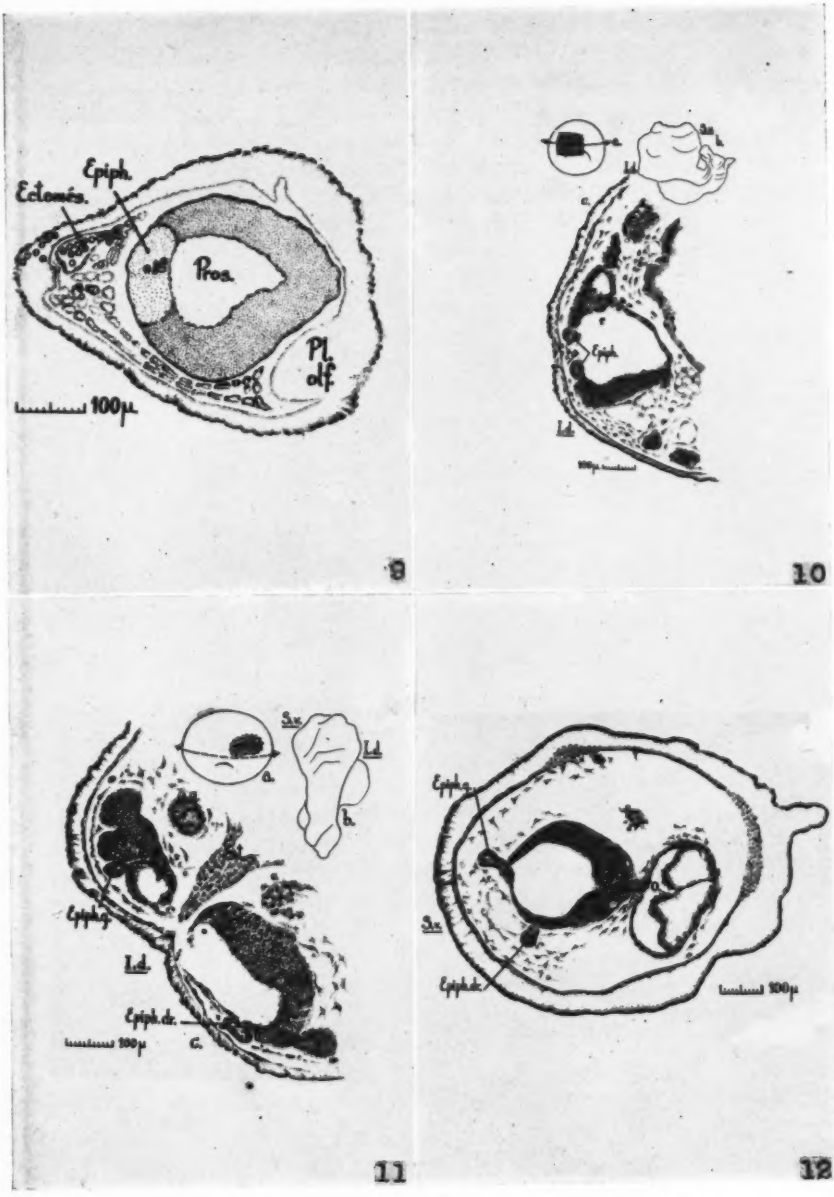


FIGURE 9
FIGURE 11

FIGURE 10
FIGURE 12

the Anura, the first appearance of a pineal body may be observed as a small swelling of the diencephalic roof, just behind the last remnant of the closing neuropore (Fig. 8). Very soon, these cells acquire a distinct cytological aspect with a lightly staining cytoplasm. An early work of Spemann ('12) pointed to the fact that the unity of the pineal body is only secondary; it takes origin from two distinct groups of cells situated symmetrically at the edge of the cerebral plate, which normally fuse by the closure of the cerebral vesicle. This process has been demonstrated by vital staining in Spemann's laboratory by A. Daltrop ('31), and has been confirmed by Van de Kamer (in Raven, '46). The double origin of the epiphysis can also be shown by a unilateral vital staining of the young gastrula (Fig. 9). Therefore, it is not surprising that several of the translocations yielded double pineal bodies (Fig. 10 to 13). They are of variable size, often each as large as a normal one, an indication of a perturbation in the inductive factors at work.

Let us now examine more closely the forebrain parts in our double embryos. Some of them, in fact, have two normal or nearly normal prosencephalons, mostly with some microphthalmia (Fig. 14). Such exceptional success seems to be related to an equal division of the prechordal anlage, with a small loss of material and a compensatory regulation. Ordinarily, differences are present, and it is not astonishing that the less one of the forebrains is developed, the more the other is complete. Nevertheless, some microphthalmia or tendency to monorhiny often appears as a symptom of a partial loss of the specific evocator.

One negative observation should be recorded. It might have been possible to observe a complementary situation between the three main auxiliary organs (eyes, olfactory lobes, epiphysis) of the two embryos, one being provided with optic and pineal structures, the other with olfactory ones. Or, on the other hand, one embryo might have larger organs on one side, the other one on the opposite side. This would have meant, or at least suggested, that the prechordal material of the blastula or young gastrula has already been endowed with a symmetrical pattern, and also with specialized evocators of the different secondary organs. Since such a possibility has been found valid for the oto-evocators, it would have been reasonable to expect it for this part of the evocatory material also. Nothing of this sort is to be found. It does not matter whether the part of the prechordal anlage

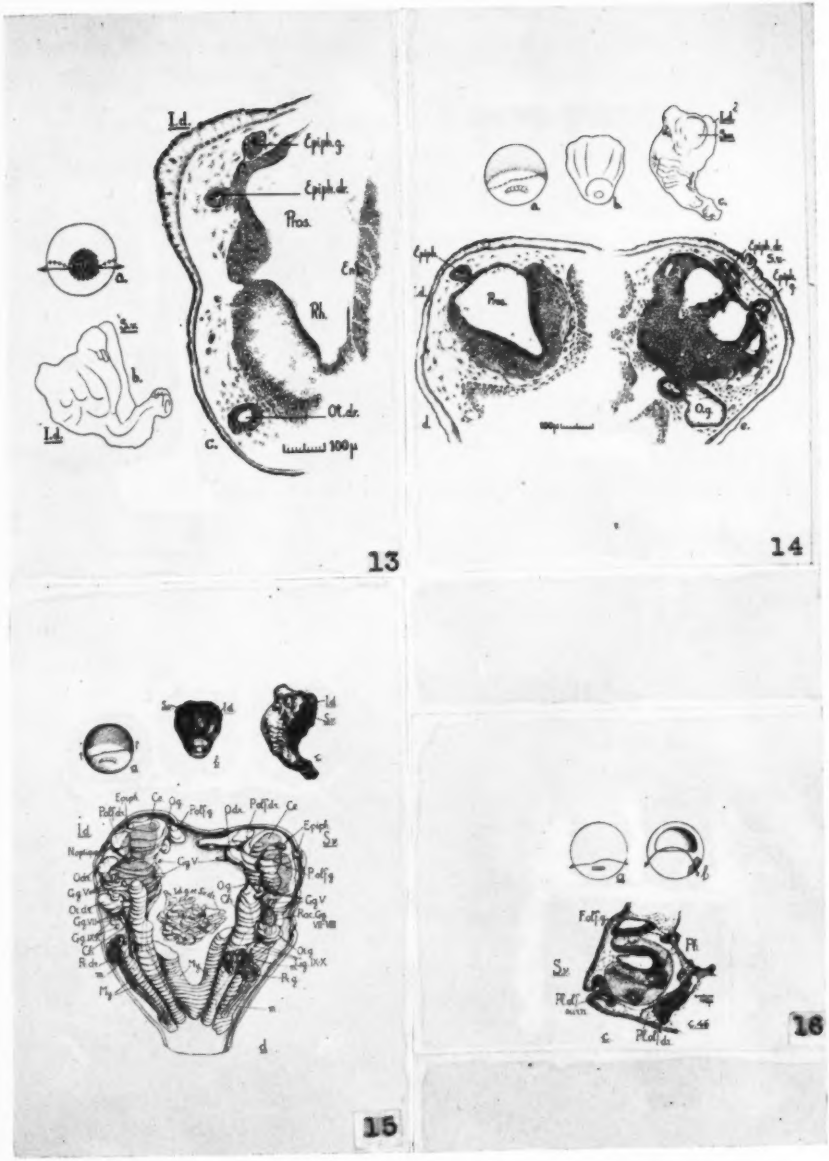


FIGURE 13

FIGURE 14

FIGURE 15

FIGURE 16

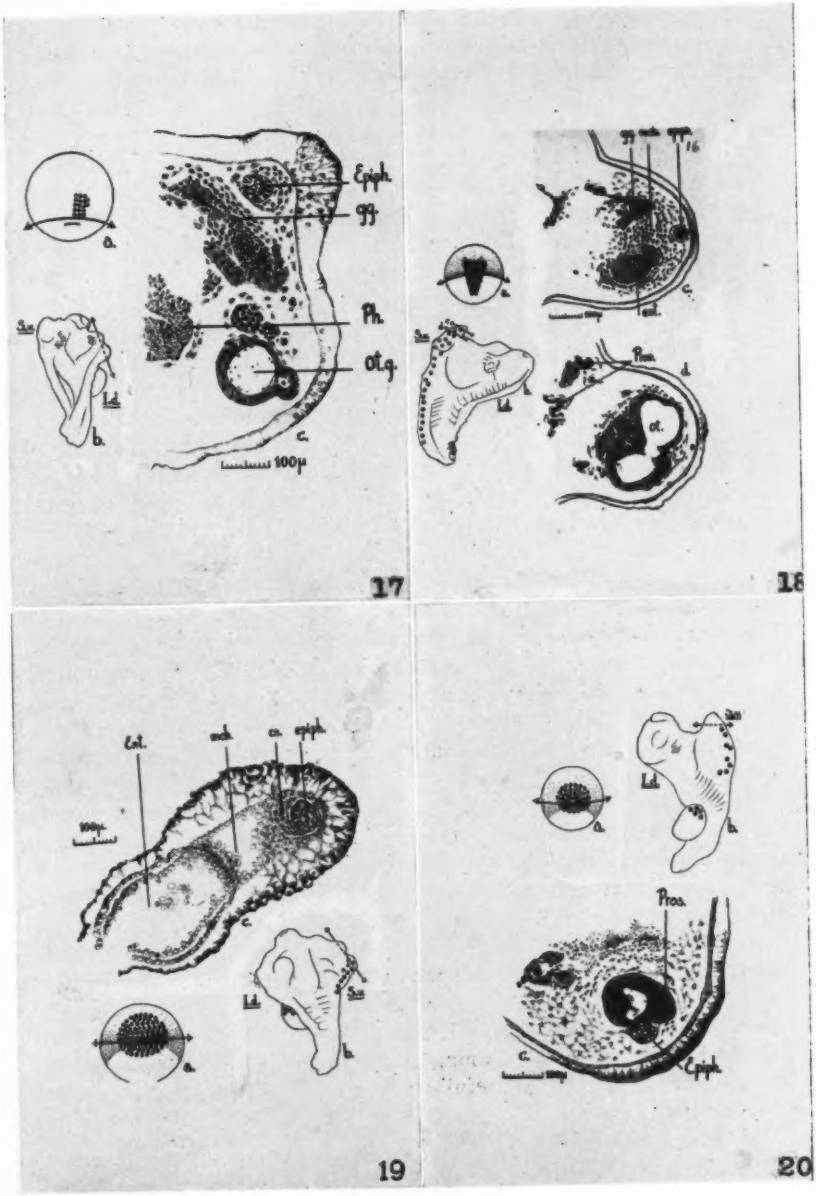
that is present is the superior or the inferior, the superficial or the deep, the lateral or the medial, its activity is always of the same nature. Besides, when an oblique section has been made which certainly separates the left and the right parts of the prechordal material, the forebrain structures develop a striking symmetry (Fig. 14, 15, 16; see details in the explanations of the figures). Hence, the idea of an early complication of the prechordal material must be rejected. The facts point, on the contrary, to its *homogeneity*, at least in the blastula and young gastrula stages.

This, however, makes it necessary to search for an appropriate explanation for the complexity of the prosencephalon, its constriction into two vesicles, and the budding of the olfactory, optic and pineal parts. The clue to this puzzling question is to be found, in my opinion, by a closer examination of the differences between the two forebrains, but from quite a different point of view.

They do not display, as has already been said, a complementary organization in their anatomical details, but they nevertheless present a correspondence in their general features. To understand the nature of that relation, the following facts are significant:

1) The manifestation of the prosencephalon can be most curiously limited to the pineal body, without any other true neural tissue, except for neural crest material which seems necessarily to accompany the epiphysis, which is otherwise quite isolated. In three double but unequally developed embryos, the smaller nervous system seems, at first sight, to be limited to a spinal cord, a reduced rhombencephalon, and two auditory vesicles (Fig. 17), or only an apical one (Fig. 18). But, if the anterior sections are attentively examined, they reveal, among dispersed mesoblastic, ectomesoblastic and ganglionic cells, a small, round body, in an exactly medio-apical position. Its structure, the character of its cells, and their delicate pigmentation, permit one to make definite conclusions as to its pineal nature.[1] (See Figs. 17, 18, 19.) Its presence is only to be understood by the assumption that in front of the reduced neural plate of the system, a localized induction has taken place. The peripheral part of this neurectoblastic material has given rise to migratory neural crest cells, homologous with the prosencephalic crest. The condensed remaining part has formed an epiphysis. Such structures may be considered as the lowest manifestation of a prosencephalon, and are supposed to be induced by a small amount of prechordal material.

[1]This may occur either in the I. d. system (Fig. 17) or in the S. v. system (Figs. 18, 19).



FIGURES 17 TO 20

2) In all more distinctly cephalized systems where pre-auditory parts of the brain have developed, the pineal body is *always* present. The olfactory and optic parts may be normal, reduced or atypical, but the pineal body is nevertheless regularly found, and is sometimes double (Figs. 20 to 23).

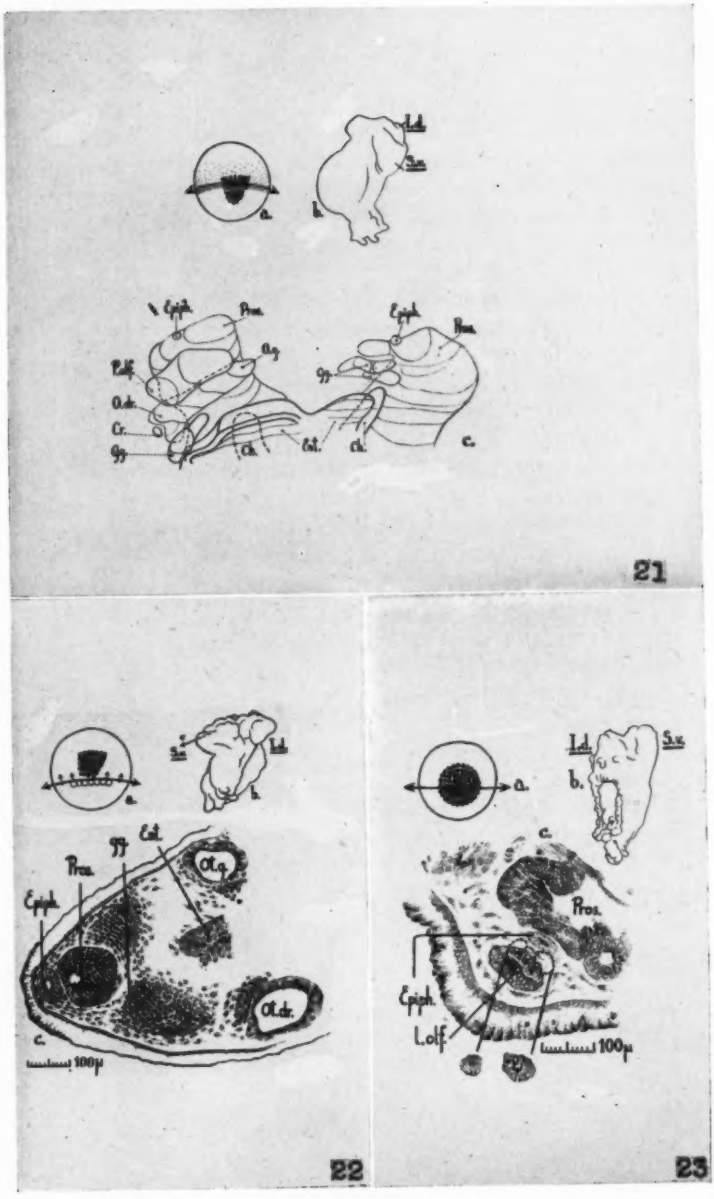
3) The defective forebrains can easily be classified in a progressive series. The first degree of differentiation is the isolated pineal body. The second is a small, vesicular forebrain, without any olfactory or optic enlargements, but, of course, with an epiphysis (Fig. 20, 21, 22). In such cases, a more extensive cerebral plate must have been induced, two epiphyseal anlagen must have been formed on its borders and become fused by the closure of the cerebral vesicle. It is reasonable to explain such a result by the assumption of a somewhat more extensive amount of prechordal material.

A third degree of differentiation is represented by a somewhat larger vesicle, which has formed a finger-like anterior expansion capable of inducing an olfactory placode (Fig. 23). This is the monorhinic degree, completed, of course, by a simple or double pineal body.

A fourth degree involves the addition of the optic structures to the preceding ones (Fig. 12). A fifth one is characterized by structural patterns ranging from monorhiny combined with cyclopia to the possession of two olfactory lobes and two optic vesicles (Fig. 14, 15, 16). When this superior level of organization is reached, it becomes difficult indeed to say whether the optic bilaterality constitutes a higher level than the dirhinia. Embryos having two normal or reduced optic vesicles generally have two olfactory placodes, but sometimes bilateral microphthalmia is associated with monorhinia (Fig. 22).

Be that as it may, it must be admitted, as pointed out above (p. 98), that there are no specialized evocators of the various forebrain organs. Hence, the only interpretation of the higher levels of forebrain organization is that these are due to larger amounts of prechordal material.

4) *Gallera's and Damas' investigations.* Because of the preceding data, I thought it probable that the *strength* and *duration* of the inductive action might be decisive for the nature of the nervous structure to be acquired by the various regions of the neural plate. That is why I suggested that in young neurulae, pieces of neurectoblast of known position should be carefully separated from the archenteric roof,



FIGURES 21 TO 23

and grafted into the ventral epiblast of similar hosts. This experiment has been performed in two Anura, *Rana esculenta* and *Xenopus loevis* and in two Urodeles, *Pleurodeles Waltii* and the Axolotl. H. Damas investigated the trunk region, G. Gallera the prechordal region.

In the first experiment, three successive pieces of tissue were isolated from the presumptive spinal territory of a very young neurula near the mid-dorsal line, without encroaching on the mesoblastic hindpart of the neural plate. When grafted on the abdomen, these pieces readily showed that their material did not necessarily develop in the same way that it would have done in situ; it is capable of *paragenesis* instead of *normogenesis* (Dalcq, '35), terms which avoid the implications involved in the current expressions of "strict" and "labile" "determination". If the explantation is performed before any cytological differentiation has begun,[1a] each piece yields a certain amount of neural crest material, which is true paragenesis. The posterior one usually becomes dispersed into pigmented cells and ganglion-like masses, the middle and anterior grafts give more and more spinal cord tissue, but with various transitional, atypical aspects which are really interesting. Briefly, *the progress of typical neural differentiation is a function of the location of the inductive material and the duration of its action.* Neuralization implies an ascent through successive levels: a first level probably corresponding to melanocytes, a second to ganglionic cells, a third to a loose spinal cord structure, a fourth to epithelial, ependyma-like cells enclosing a lumen, and so on. There is a definite *cephalo-caudal decrement* of the inductive strength of the archenteric roof, just as there is a similar *dorso-ventral decrement* which is responsible for the different fates of the medial, medio-lateral and lateral parts of the medullary plate. That is probably[2] the situation in all of the chordal part of the medullary plate.

In regard to the prechordal part of the cerebral plate, two analogous experiments have been performed by Gallera. Because of the well-known acceleration of the morphogenetic events in that anterior region, it is necessary to operate on younger stages, i.e., late gastrulae with an open blastopore.

[1a] It is possible that some paragenesis might occur, under similar conditions, at later stages, but this point has not yet been examined.

[2] The chordal region of the presumptive brain has not yet been investigated in this way. But the work of Raven and Kloos ('45) seems to justify the generalization (see footnote p. 105).

In a first series, three consecutive pieces were explanted from the midline to the abdominal epiblast. The first one usually differentiates into a large forebrain vesicle with a thick ventral and a thin dorsal wall, and some neural crest material. In several instances—a variation probably related to slight differences of stage—a well differentiated optic vesicle is formed (but never two vesicles!) sometimes with a lens of neural origin. One or more pineal bodies are observed on the dorsal side, but as yet no olfactory lobe has been found. Such forebrain structures show a definite symmetry, which is not astonishing, since the grafts were obtained from the midline. It may be said that we are dealing with paragenesis, but with great tendencies toward normogenesis. The second piece can react in a similar way, though more frequently it produces only a mass of brain tissue with abundant neural crest material. In the case of the third piece, the paragenesis is still more pronounced, as it is dispersed almost completely into ganglionic, and, for the most part, pigment cells.

A second set of operations has been of exactly the same type, except that the three grafts were excised along the medial margin of the neural fold of the cerebral region, but not including it. The results are essentially the same, but the remarkable thing is that the forebrain (or brain) parts have a definite bilateral symmetry in spite of their lateral origin.

Thus, if the explantation is performed at the proper stage, the same fundamental gradation in the level of neuralization appears as in the spinal cord experiments of Damas. Here again *neural organization and differentiation is a function of the intensity and the duration of the induction.* The explanation of certain local structures, such as the pineal and the optic lobes, is nearly at hand; these probably develop by a succession of events which we hope Gallera will be able to elucidate in the near future. The difficulty with these anterior territories is that the evocating process takes place with great rapidity. Nevertheless, the possibility of altering the fate of the neurectoblast by stopping its induction at a certain moment is henceforth established. So also is the fact that *the merely quantitative conditions of the induction confer on the neurectoblast intrinsic properties controlling bilateral organization.*

III. Interpretation

The many questions arising on brain formation concern its general nature and its specialized pattern. Their solution can only be approached when they are discussed from the point of view of general morphogenesis. When we endeavor to explain such a fundamental biological process, our thoughts necessarily follow some well-worn path, starting from the classical dilemma, *Preformation* versus *Epigenesis*. In the present analysis, as in many others, this old distinction may be useful as a starting point, providing that we realize its merely relative and mainly superficial value.

When we limit our consideration to the archenteric roof of the late gastrula and young neurula, the respective rôles of preformation and epigenesis may easily be distinguished, thanks to Spemann. Preformed are the intrinsic properties of both middle and upper layers, that is to say, the organization proper to the first and the competence of the second. Epigenetic are the inductive effects exerted by the underlying layer on the ectoblast.

1. *The epigenetic events.* Induction will not be considered here from the fascinating point of view of its biochemical nature, but in its relation to the subsequent segregations of neural parts and sense organs. In principle, we must account for the segregation of true neural tissue and neural crest, a process common to the entire neurectoblast, for the preponderance of the cerebral over the spinal region, for the evocation of the auditory placodes, and finally for the modelling and differentiation of the specialized forebrain and hindbrain structures.

A. *Neural crest and neural tissue.* In a theoretical essay formulated with Pasteels in 1937, we have suggested an explanation of primordial morphogenesis on a mainly quantitative basis. This point of view led us to consider the neural segregations as principally related to quantitative differences in the inductive process. More specifically, in 1938 and 1941, I stressed this mode of explanation for the segregation between neural crest and neural tissue.

This conception has recently been submitted to an experimental test by Raven and Kloos ('45). After grafting the medial and the lateral parts of the archenteric roof (without ectoblast) separately into the blastocoel, these authors concluded that only a quantitative

difference in inductive power was to be detected between these grafts.[3] The same point of view has now been confirmed more directly by the recent experiments of Damas and of Gallera. The normal differences in evocating power which are linked with chronological and topographical relations can be imitated by the interruption, at a given moment, of the inductive action. Thus, the solution is pretty clear with regard to segregation in a transverse, dorso-ventral direction.

B. *Cephalo-caudal differences—Prechordal individuality.* For the cephalo-caudal direction, the situation is indeed more intricate.

On the one hand, several facts speak in favor of a simple cephalo-caudal decrement all along the archenteric roof, its pre-chordal part included. With LiCl intoxication, it is now established beyond any doubt (Pasteels, '45) that the degradation of the structures starts with the apical, prechordal region, and gradually extends towards the caudal levels. In Damas' and Gallera's experiments, the differences of paragenesis clearly reveal the same cephalo-caudal decrement. The results of Nieuwkoop also point in the same direction. The gradations observed in his secondary embryos with regard to the completeness of their cerebro-spinal axis seem to have been caused by involuntary variations in the amount of grafted material, perhaps by slight injuries to it, more probably by the degree to which the organizing properties were transmitted to the surrounding ventral mesoblast. Nevertheless, it is certain that the blastoporal lip contains mainly the head inductors: when only a spinal cord, or hindbrain structures in addition, are obtained, the prechordal plate must have been included in the graft, at least its superficial part. If it is endowed with a real specificity, the prechordal material must have lost it under these conditions, in the same way as when it is grafted into the advanced blastoporal lip (Hall, '37). Again, when younger stages are considered, the egg inversion experiments even provide arguments for the possible transformation of trunk-inducing into head-inducing material (Pasteels, '39).

On the other hand, most of the microsurgical operations performed on the blastula and gastrula stage (cf. p. 94), together with my own translocations, definitely point towards a relative individuality of the prechordal anlage. One of the results mentioned above (Fig. 6 and 7) is, in my opinion, especially significant. Gallera's results also show

[3]These explants were taken from the hindbrain region, which makes it likely that the explantation of that material will give results similar to those of Damas (p. 102).

in a striking way how the effects of prechordal induction are on a higher level than those in the spinal cord region. Of course, as has been explained (p. 198), there is no clear cut difference. Certainly adjacent material is more or less able to increase its activity to the prechordal level. But it would be exaggerated to speak of determination, or of a proper specificity. The situation can only be pictured with such a cautious term as "predisposition" (Dalcq, '35), or "inclination".[4]

This is, however, not entirely confined to the prechordal material. At the blastula stage, and perhaps much earlier, the amphibian embryo reveals a distinct tendency to develop into an intricate constellation of predisposed territories. Latitudinal translocations demonstrate it for the presumptive notochord, somites, pronephros anlage, coelomic material, pharyngeal entoblast and some minor regions (Dalcq, '41). There is a characteristic tendency towards emancipation. But, in case of emergency, the labile individuality is sacrificed to the general obedience.

With these reservations, we cannot escape the conclusion that the prechordal anlage possesses some early quality, still labile, which can easily be imagined in general biochemical terms. This is also the only way to understand how certain xeno-inductors of various origins are all able to induce the formation of typical forebrain structures (Chuang, '39, Toivonen, '40). In my opinion, such facts might be attributed to the action of certain substances which happen, by a kind of biochemical convergence, to have the same evocating properties as the prechordal material.

C. *The oto-evocators.* A similar conception may be inferred with regard to the oto-evocators. They have in the blastula, and hence in the gastrula stage a sufficient degree of individuality to be divided by appropriate sections and have their parts displaced, at least together with their immediate surroundings. But, to a somewhat greater extent than the prechordal material, they are likely to be replaced in their function by other elements of the gastrula, most easily by head mesoblast, sometimes also by remoter material. As we regard the specialized aspect and mode of differentiation of an auditory vesicle, we might feel inclined to consider that its evocator must be of a distinctly specialized biochemical nature, in comparison, for

[4]Term used by Dr. Yntema.

example, with the evocator of an olfactory placode, the source of which, as is well known, is the olfactory lobe. Such a view is again exaggerated, as in one translocation I happened to observe the replacement in situ of an olfactory placode by a typical otocyst. Moreover, the induction of an otocyst may be effected by various living or other agents, especially by neural crest material (Trampusch, '41). In my opinion, this last induction is analogous with the homogenetic induction exerted by the neural tube itself (Spemann and Mangold, '27), whereas, for the other cases, some sort of "convergence" effect should be postulated. In any case, I am not inclined to admit that the rhombencephalic crest is the *normal* oto-evocator. The translocations afford structural dissociations which exclude that hypothesis. I do not see any positive reason for abandoning the position I adopted in 1933: the oto-evocator is a group of cells located in the parochordal mesoblast. Zwilling ('41) has arrived at the same conclusion in a more recent investigation.[5] C. L. Yntema ('46) is also of the same opinion.

D. *The field of inductive activities.* When all these considerations are taken into account, the epigenetic events of neurulation can be understood in the following way. The whole archenteric roof (sensu stricto) represents a field of inductive activities having its main point slightly cephalad to the apex of the future notochord. From there, it presents a decrement, for a short distance, in anterior direction and a longer and smoother one backwards. Moreover, a bilateral decrement is grafted on each level of the cephalo-caudal axis. This general disposition of the field accounts for the appearance of neural and crest tissue. It is now completed by three regional specializations. The inductive power is increased in both symmetrical regions of parachordal mesoblast, which, while not capable of forming true somites, nevertheless produce a high concentration of inductive substances. This causes the enlargement of the posterior half of the cerebral plate, and also, through the operation of a still more concentrated evocator, the induction of the auditory placodes. Notochord and

[5]Microplacodes have been neglected in that discussion. One would probably be tempted to consider the corresponding neural crest as the agents of their induction. There is, however, at least one experimental result which is not in accord with that idea. In the chick, the suppression of one half of the brain and of its neural crest does not hinder the formation of the microplacodes. Here also, per exclusionem, mesoblastic agents seem to be responsible (E. van Campenhout, '35. *C. R. Soc. Biol.*, **118**, 1653-1654).

parachordal mesoblast are in this way responsible for the hindpart of the brain, which has been referred to as the chordencephalon (p. 87).

Finally, the anterior part of the archenteric roof is functionally (and qualitatively?) reinforced in the region of the prechordal plate. Here, if my interpretation is correct, the induction can never give purely neural tissue. If it reaches the cerebral level, a pineal body is also formed, together with the inevitable neural crest. If not, the induction provides for an isolated epiphysis. Such a functional particularity is probably related to the incipient biochemical specificity already mentioned. If the cerebrogenous level is again surpassed, the acrencephalic ampulla forms its olfactory expansion first, and then its optic evagination, if the attained level is sufficiently high.[6]

This, in my opinion, is one of the morphogenetic events in which the importance of quantitative differences is most remarkable. No organs are more apt to give us a direct impression of intrinsic qualitative differences than the eye, the olfactory pit and the pineal gland. And yet, the idea of relating them to specific inductors must be rejected—latitudinal translocations justify us in stating that all parts of the prechordal anlage of the blastula and of the young gastrula are *commutable*. This may be understood in a relatively simple way: When the condensation of the cells is greater, the elaboration of the inductive substances is more intense. As this plate of cells is gradually flattened towards its borders, its periphery would be able to evoke only prosencephalic neural crest, a structure well known to be only transitory. A certain lateral region would attain a somewhat higher activity, corresponding to the epiphyseal body. Its induction is remarkable in that the effects become evident only after a period of latency; it may be called a *deferred induction*, with the remark that the distinction between immediate and deferred induction may be useful to further investigation. In the case of the epiphyseal anlage, a correlation may be suspected with the fact that the normal closure of the neural tube shortens the duration of the induction exerted by the lateral areas of the prechordal plate. The more internal region of this material would elaborate more concentrated substances, which, acting longer, would lead to true and immediate cephalization. A still more intense action in the central region would provide for the modelling of the olfactory lobe or lobes anteriorly, of the otic vesicle or

[6]The correlations responsible for the modelling of the infundibulum are not considered in this discussion.

vesicles posteriorly. The reason why these organs remain single or become double will be considered in the third part of this discussion.

E. *The explanation for the neural deficiencies.* The experimental reductions of the cerebro-spinal axis which are of interest for our morphogenetic problem are threefold: those obtained by LiCl intoxication (literature in Pasteels, '46); those attained by complete and deep division of the dorso-marginal zone, by the method of translocations; those observed by grafting a definite part of the same material, as in many experiments on the organizer, and especially those of Nieuwkoop.

The results of the two first groups are in remarkable agreement for the head as well as for the notochord and somites. The mass reduction of the dorso-marginal material has effects which are very similar to those obtained by the degradation of the "morphogenetic potentials" with the use of LiCl.

With grafts of the blastoporal lip, the situation is not exactly the same. A given amount of normal chordo-mesoblast (sensu largo) does not become stretched into a complete but thinner archenteric roof, quantitatively deficient in all its parts. Under these conditions, it would induce a regularly reduced cerebro-spinal axis, with a main deficiency effect on the higher levels, and a gradually fading one at the lower levels. On the contrary, the available material does its best to act as it does under normal circumstances, and then stops abruptly. Important reciprocal cell correlations must be effective in that process: factors controlling the normal aggregation of the cells which thereby insure the gradients their normal slope.

Taken as a whole, Nieuwkoop's data demonstrate very clearly that the development of the nervous system is under the guidance of a gradient of morphogenetic potentials proceeding headward. The more powerful the flow of invaginating chordomesoblast, the farther it migrates under the ectoblast, and the sooner its anterior parts get into position where they exert their inductive activity, the higher will be the level of structure attained by the apical part of the nervous system. In accordance with the action of these combined factors, it develops up to the level of some anterior spinal nerves, or of the last cranial pairs, or of the mesencephalon, or even of the di- or telencephalon.

The possibility of an apical mesencephalon is of interest. In my previous publications on this problem ('46, in press), I supposed the

mesencephalon to be simply a kind of copula between acrencephalon and chordencephalon. The individuality of the midbrain may be more marked, if the organization described by Nieuwkoop is related to primary inductive activity and not to internal correlations that are linked with neurological differentiation, a possibility which has not been excluded.

Finally, while it is true that a general seriation of the deficiencies demonstrates a headward progression of the morphogenetic levels, this direction of the gradient does not hold for the forebrain region. Optic structures are certainly not so easily formed as the olfactory ones, and are more sensitive to debasing agents. As the optic anlagen are posterior, it must be supposed that the caudo-cephalic gradient attains in that region a point of inflexion. This is another indication that the prechordal part of the inductive field has a different organization than its chordal region.

F. *Neurogenous induction and growth.* It is apparent that the gradual transformation of the neurectoblast implies a considerable synthesis of protoplasm. The conformation of the spinal cord and of the brain is mainly related to general growth differences and regional intensifications. The inductive field with its general pattern and its local intensifying factors does not only call forth cytological differentiation, but also protoplasmic synthesis. In the brain, the *deferred induction* mentioned above is probably of considerable importance for later growth. The secondary development of certain dorsal parts, namely, the cerebellar and the cerebral hemispheres, may be related to the originally lateral location of those materials. In their case, deferred induction should be exclusively growth determining, and not properly morphogenetic, which is an interesting dissociation.

As J. Brachet ('40, '44) has been able to show by important investigations, the growth effect of induction is related to the SH protein compounds and to the nucleic acid metabolism. These processes are the earliest and the most intense in the prechordal region. The grafts made by Gallera demonstrate this in a striking way.

This point of view also provides, in my opinion, the main interest of Trampusch's irradiations. Of course, this author is quite right in emphasizing that his results are largely explained by the injurious effects of the X-rays on the prechordal anlage. But it also seems important that the general pattern of the brain has been maintained without monorhiny or cyclopia, but with a considerable reduction of

size. Moreover, the difference between these results and those of later irradiations is really striking. It is probable that the effects of irradiating have to do largely with nucleic acid metabolism. If it were possible by means of appropriate screens to irradiate the dorso-marginal zone and the presumptive neurectoblast separately, a new field of investigation would be opened. We can be confident that the author of that original contribution will not deceive our hopes.

IV. The Preformation Aspect of Neurogenesis

Two components play a part in this process: the middle and the external layer. Both are indebted to previous processes for the properties which make the first the inductor, the second the reactor. They will be dealt with separately.

1. *The formation of the inductive system.* The pattern of presumptive organs and of inductive activities present in the archenteric roof is only the intensified expression of the predisposed territories detectable from the blastula stage. The origin of these predispositions has been discussed in our "Field-gradient-threshold Theory" (Dalcq and Pasteels, '37, '38; Dalcq, '38, '41). Without going into a detailed account, it may be of some interest to mention our present point of view concerning that hypothesis. Experiments performed during the last two years by my distinguished co-worker have constantly resulted in confirmations of the existence of the cortical field. He has been able to refute decisively[7] the opposed thesis of Lehmann ('42), which argues for a "marginal plasma" pre-existing in the fertilized egg. In spite of the difficulties inherent in a crucial demonstration, we feel justified in asserting that the cortical field is a reality.

As we have previously pointed out, its morphogenetic role is only to be understood if we accept the thesis that a reaction occurs between the cortical film and the internal material. We suggested that this internal material was constituted by the yolk platelets, or substances derived from them. We tried to explain the fate of the various regions by the combined effect of the local intensity of the cortical field and of the yolk concentration in the underlying cytoplasm. We admitted, in accordance with the current classical view, a primarily continuous yolk gradient from the animal to the vegetal pole. On this last point, however, some improvements of the theory are necessary.

[7] *Acta morphologica helvetica,* in press.

The regular yolk gradient assumed by us is not a reality. Peculiarities of yolk distribution, rightly pointed out by Lehmann ('45, p. 182), must be taken into account. An observation furnished by the interesting experiments of Ancel and Vintemberger ('44) has recently drawn Pasteels' attention to a special crescent-shaped layer of middle sized yolk platelets, which is responsible for the well known gray crescent and, in inverted eggs, for the Born crescent. This may have much to do with the organization of the dorso-marginal zone. On the other hand, local concentrations of yolk may be disturbed without involving distinct consequences for morphogenesis. And yet, large yolk perturbations evidently play a decisive rôle. It seems that the general pattern of the morphogenetic organization depends on a mass effect of the concentrated vegetal yolk, while the marginal yolk crescent has a more local effect. In any case, the idea of a cortico-internal reaction, with a whole system of quantitative gradations resulting in a morphogenetic field, remains the best clue to a logical interpretation. The core of our theory is perfectly sound, and will permit us, in the near future, to integrate the results of new investigations into a still more satisfactory concept.

Of course, it is necessary to account for the quantitative differences between the embryonic territories, and to explain their gradual emancipation and segregation by the general biological principle of functional thresholds. But sooner or later, qualitative differences, based on biochemical metabolism, will necessarily arise, and this new step, outside of its general interest, has a direct importance for our problem, given the relative individuality of the prechordal material. At this critical point, the only reliable clue seems to be an invocation of the genes.

The numerous arguments in favor of their morphogenetic action have often been discussed, and I paid due attention to them in my book of 1941. But, with regard to prechordal activity, it is perhaps worth mentioning that a gene located in the X-chromosome is known to be responsible for human microphthalmia (J. A. Frazer Roberts, '40). If the concept here defended may be generalized, such hereditary deficiency must also depend on a reduction of the inductive power of the prechordal plate of the affected human embryo. This fact is therefore a new indirect argument in favor of the idea that a qualitative morphogenetic pattern is superimposed on the quantitative one by the initiation of some genic activities.

2. *Ectoblast competence and eventual reacting field.* In considering the reactor, we must again examine its general and regional properties.

The general properties simply concern the *competence* (Waddington) of the tissue. Thus far, its rôle has been recognized instead of analyzed. It is easy to see which developments are at hand within the type of experimentation initiated by my co-workers Damas and Gallera.

Special regional properties have often been claimed to exist in the presumptive neurectoblast (e.g. Barth, '41) but so far they have not been clearly demonstrated. Nieuwkoop introduces the somewhat different view that the reacting ability of the ectoblast to the inductor decreases from the animal pole to the equator. This hypothesis does not seem indispensable to the interpretation of his results. Nevertheless, it makes them easier to understand, and it may be argued that such a peri-animal gradient has been observed in the fertilized egg for the ribo-nucleic and SH protein components (J. Brachet, loc. cit.). To confirm Nieuwkoop's conceptions, rotations of dorsal territories are easy to plan and to perform. Should they be negative—and I fear they will—the idea need not be abandoned without further analysis. If a microchemical field were detectable in the roof of the blastocoele as it is in the fertilized egg, such a field would eventually be likely to be restored in spite of the rotations performed to change its organization.

3. *On the acquisition of bilateral symmetry.* If the interpretation defended here is correct, especially as regards the points concerning prechordal induction, it is only a quantitative relation which determines whether or not the olfactory and the optic apparatus will attain a symmetrical arrangement. Transitions between the arhinia with anophthalmia, monorhinia with cyclopia, presence of two olfactory lobes and two optic vesicles are so gradual that no special factor can be imagined to control symmetry.

This statement calls attention to the need for an explanation of the symmetrical organization of the vertebrate body. Indeed, thus far, only asymmetrical conditions have attracted attention, the symmetrical condition usually being considered inherent and self-explanatory. For the purposes of the present discussion, we perceive that the symmetrical arrangement is linked with a certain degree of concentration of the inductive substances.

Is the first step towards symmetry taken within the evocator, or within the reacting system? The first of these possibilities seems to have been the accepted one, at least implicitly (Adelmann, '36). But, on the one hand, it may be stated that no sign of bilateral arrangement is visible in the prechordal materials at stages when the bilaterality of the forebrain and the localization of the sense organs is already evident (Fig. 4, 5). On the other hand, Gallera's grafts indicate that the tendency to bilateral symmetry is intrinsic in the newly induced neurectoblast. Another striking argument is afforded by the embryo shown in Fig. 16. The olfactory lobes are unequal, and the larger one has induced a supplementary and just medial olfactory placode. Obviously, no symmetrical organization exists in the inducing lobe, and yet the placode has acquired a distinct bilateral disposition. This makes it clear that no occult bilaterality needs to be postulated in the substrate to explain the bilaterality of the reactor. *If the inductive action is sufficiently powerful, it confers to the reactor the factors governing the bilaterality of the latter, and these are factors of localized modelling and growth.*

Since our attention is directed towards such relations, it will readily appear that they are susceptible to extensive generalization. When it happens that the oto-evocators form the apex of an inductive system, they do not evoke a large, single otocyst, but a bilocular one (Fig. 18). When abnormal oto-evocators are stretched into a long strip (Dalcq, '33), the induced vesicle is never a giant one, but becomes plurilocular, each enlargement being approximately the size of a normal otocyst. When supplementary notochordal material is grafted into the notochordal field of a gastrula, no giant notochord is obtained, but two more or less separate ones. When the chordo-mesoblastic primordium of a gastrula is greatly reduced in amount, as in many of my translocations, it does not yield a miniature notochord with reduced somites, but a notochord surrounded by semicircular, medial somites.

The general interpretation of these facts appears to be that there exists, for each organ, a *size limitation,* which is presumably imposed by mutual cell correlations. When the number of elements exceeds the limit compatible with an organ unit, a rearrangement intervenes, and symmetry is acquired. The point is, that in normal embryos, morphogenetic substances are provided in such a way as to insure bilaterality for all primordial organs excepting the axial ones, the prechordal, chordal and entoblastic anlagen. It is very probable

that spontaneous double monsters are due to an excess of the precursors for dorso-marginal materials, so that two separate morphogenetic fields have to be formed.

What we seem justified in thinking concerning primordial organs is only an extension of the relations at the less complicated levels of cellular organization. The division of chromosomes, nucleoli, centrosomes, chondriosomes and of the cell itself has in my opinion not received any other explanation than the existence of a limit of size for each type of organelle. This may be considered a law of biological organization.

V. Summary and Conclusions

1. The questions raised by the morphogenesis of the brain are formulated.

2. Recent data concerning this problem are described:
   a) irradiation experiments by Trampusch
   b) grafts of the blastoporal lip by Nieuwkoop
   c) results of latitudinal translocation related to the induction of the hindbrain, forebrain and otocysts
   d) unpublished results concerning the explantation and grafting of neurectoblast which has been submitted to inductive influences of short duration (Damas, Gallera).

3. It is assumed that the unity of the cerebro-spinal axis is only apparent. The neural plate results from the combined action of (a) the notochordal part of the archenteric roof, (b) the parachordal mesoblast (with the oto-evocators), (c) the prechordal plate.

4. Two main parts are to be distinguished in the brain: *acrencephalon* (archenkephalon of Lehmann) and *chordencephalon* (deuterenkephalon of the same author).

5. The degree of individuality of the prechordal plate and of the oto-evocators at the blastula and gastrula stages is discussed. A distinct individuality is proposed, which however is still labile and capable of being substituted by adjacent material.

6. The prechordal plate does not contain specialized evocators for the prosencephalic neural crest, cerebral tissue, pineal body, olfactory lobes or optic vesicles.

7. The lowest manifestation of a prosencephalon is an isolated pineal body. It corresponds to the presence of a minimal amount of prechordal material.

8. More complicated structures of the prosencephalon are correlated with higher levels of induction. They form a hierarchical system.

9. The pattern of inductive activities in the archenteric roof is described in terms of field and gradient. Quantitative relations are fundamental, though reservation is made for the relative specificity of the prechordal region.

10. The acquisition of a symmetrical arrangement in the forebrain structures is caused by a sufficiently high level of induction.

11. The tendency to bilateral arrangement is intrinsic to the induced neurectoblast.

12. The bilaterality of most primordial organs is governed by a law of size limitation, which seems to have a general application in animal organization.

EXPLANATION OF FIGURES

FIG. 1. Latitudinal translocation at the blastula stage. (a) dorsal view of the embryo, with the large colored mark (small circles), and the upper row of larger blastomeres, indicating the level at which the blastoporal lip will be formed; (b and b′) animal and vegetal view of the healed embryo, at the circular blastopore stage; (c) neurula stage; (d) the embryo just before fixation.

FIG. 2. Translocation in a young gastrula. (a) dorsal view of the embryo with an asymmetrical mark (circles); it was attempted to section the embryo at the inferior limit of the notochordal field; (b) schematic sagittal section, to show the direction of the section in the deeper parts and on the ventral side; (c) the healed embryo at the circular blastopore stage; (d) the double embryo; in the sections, the S.v. system is normal, the I.d. system contains a small notochord, myotomes, a small rhombencephalon, an apical otocyst.

FIG. 3. Latitudinal translocation similar to that of Fig. 1. Reconstruction of the double embryo from the left side of the main system (the S.v. system). A low section, leaving mesoblastic material only in the I.d. system. Development in the I.d. system of mesoblast (*mes.*), a spinal cord (*m.*), two small auditory vesicles (*ot.*), and ectomesenchyme (*e. mes.*). *Ch.*, notochord; *ent.*, entoblast; *Int. term.*, terminal intestine; *my.*, myotomes; *u.*, pronephric ducts.

FIG. 4. Sagittal section of an anuran neurula, with an enlarged representation of the cephalic region. *Arch.*, archenteron; *Ch.*, notochord; *Ent.*, entoblast; *Mesobl.*, mesoblast; *P.pr.*, prechordal plate; *r.a.tr.*, transverse anterior ridge of the brain plate.

FIG. 5. Same object as in Fig. 4, but in transverse section. *C.n.*, neural crest; *Pros.*, prosencephalon; other abbreviations as in Fig. 4.

FIG. 6. Translocation dealing with a part of the prechordal material. (a) dorsal view of the operated embryo, a slightly advanced gastrula; (b) the embryo at the late neurula stage; (c and d) two transverse sections of the S.v. tubercle. *Ent.*, entoblast; *ep.dy.*, small ventricular cavity of the rhombencephalic (?) part; *Epiph.*, pineal body; *Mes.*, reduced mesencephalon (1); *Pl.olf.*, olfactory placode; *R.opt.*, stalk of the optic vesicle; *s.bl.*, patch of fibrillar substance.

FIG. 7. Graphic reconstruction of the S.v. system in the embryo of Fig. 6. The organs are seen from the right side. *Ent.*, entoblast; *ep.*, epiderm; *epiph.*, pineal body; *Inf.*, infundibulum; *m.*, short spinal cord; *mes. ect.*, mesectoblast; *Pl.olf.* olfactory placode; *Pl.x.*, supplementary (olfactory?) placode; *Pr.*, prosencephalon; *ves.* (*aud.*?), small, perhaps auditory vesicle; *v.opt.* (*rud.*), rudimentary optic vesicle; *v.s.h.*, subhepatic vein.

FIG. 8. Sagittal section of an Anuran brain. *Ch.*, notochord; *Ent.*, entoblast; *Epiph.*,

epiphysis; just cephalad to it, two asterisks indicate a small, remaining portion of the neuropore; *Inf.*, infundibulum; *Mes.*, mesencephalon; *Pros.* prosencephalon; *Rh.*, rhombencephalon.

FIG. 9. Transverse section of the head of a Discoglossus embryo developed from a gastrula which received a vital stain mark on its ectoblast, to the left of the mid-sagittal line. The epiphysis primordium (*Epiph.*) is distinctly clearer. The vital stain (circles) is found in the epidermis, the mesectoblast (*Ectomes.*—partly right) and the left half of the pineal region, which is clearer. *Pros.*, prosencephalon; *Pl.olf.*, olfactory placode.

FIGS. 10 TO 13. Four cases of double pineal body observed in one of the double embryos. Figs. 10, 11 and 13 show it in an I.d. brain, Fig. 12 in a S.v. brain, where it is associated with cyclopia (*o.*). *Epiph. dr.*, *Epiph. g.*, right and left pineal body; *o.g.*, left optic vesicle; *ot.dr.*, right otocyst. Other abbreviations as above.

FIG. 14. Translocation in a blastula with the formation of two complete brains. (a) dorsal view of the embryo; the two ? indicate that the obliquity of the section was not noted on the protocol, but could be deduced from the fact that the two systems were secondarily brought side by side, and from the repartition of the trunk organs, the large upper blastomeres (blastoporal level) having been drawn up. (b) the neurula stage; (c) the double embryo; (d) graphic reconstruction seen from the common ventral side, the digestive tract not being represented. *Ce.*, brain; *Epiph.* pineal gland; *C.g.*, cranial ganglia; *N.opt.*, optic stalk; *P.olf.dr.*, *P.olf.g.*, right (or left) olfactory placode; *O.dr.*, *O.g.*, right (or left) eye; *Ot.dr.*, *Ot.g.*, right (or left) auditory vesicle. Other abbreviations not necessary in this paper.

FIG. 15. Oblique translocation in a blastula. (a) Dorsal view of the operated embryo, with the row of upper, large blastomeres (blastoporal level); (b) the neurula; (c) the double embryo, with the two systems brought side by side (usual cinematic effect of oblique sections); (d) section through the I.d. head, with normal brain; (e) section through the S.v. head, with a brain that is also symmetrical, but affected with microphthalmia (*O.g.*, left eye visible in this section) and with two large pineal bodies. Abbreviations as in previous figures.

FIG. 16. Oblique section of a blastula. (a) Dorsal view of the embryo, the blastoporal level indicated by the row of large blastomeres; (b) schema of a sagittal section of the operated embryo, to show the course of the section in the deeper parts of the embryo; the dotted area indicates the primordia of the upper blastoporal entoblast, the parachordal material and the notochordal material; (c) section through the S.v. (main) embryo at the olfactory level. Slight asymmetry of the olfactory lobes. The larger one has induced a supplementary olfactory placode (*Pl.olf.surn.*) which is however symmetrical. *Ph.*, pharynx; *Pl.olf.g.*, *Pl.olf.dr.*, left and right olfactory placodes.

FIGS. 17 TO 19. Three translocations resulting in isolated pineal bodies. (a) Dorsal view of the operated embryo; (b) the double embryo, showing the level of the sections shown in (c) and (d). *C.n.*, neural crest material; *mch.*, mesenchyme; *Pron.*, pronephros. Other abbreviations as in preceding figures.

FIGS. 20 TO 22. Three translocations at the blastula stage, resulting in double, unequal embryos. The smaller system has a simple vesicular prosencephalon, with a pineal body. Abbreviations as in the preceding figures.

FIG. 23. Translocation at the blastular stage. I.d. system with an anophthalmic prosencephalon and double epiphysis. *L.olf.*, olfactory lobe (the olfactory placode is in a preceding section).

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